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Epi-Genetic Abnormalities of Infantile Hemangiomas: Regulation of the IGF2/H19 Locus

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Reykjavik aside, science is not performed on an island. Fortunately, I've been privileged to learn from a worldly community of researchers whose expertise are rivaled only by their generosity.

ABSTRACT

Epi-Genetic Abnormalities in Infantile Hemangiomas: Regulation of the IGF2/H19 Locus

Brent Schultz, Ruth Halaban, Elaine Cheng, John Persing, and Deepak Narayan

Purpose: To investigate the epi-genetic regulation of Insulin Like Growth Factor 2 (IGF2) and it's reciprocally imprinted transcript H19 in infantile hemangiomas.

Introduction: Infantile hemangiomas (IH) are the most common childhood tumor of the head and neck. Despite their prevalence and potentially morbid sequelae, little is known regarding the pathogenesis of this disease. However, a tumorigenic molecule, Insulin Like Growth Factor 2 (IGF2), has been implicated by microarray and confirmatory Real-Time PCR studies. There is substantial documentation that methylation abnormalities within the IGF2 and neighboring H19 loci are related to the overproduction of IGF2 in many distinct tumor types. An investigation of the methylation status, of this region, as well as the factors modifying methylation, may explain pathologic IGF2 overproduction in hemangiomas.

Methods: Using bisulfite specific methylation sensitive PCR with quantitative pyrosequencing, confirmatory genomic southern analysis, and quantitative RT PCR the methylation status of multiple regions within the IGF2/H19 locus were correlated with two potential transcriptional consequences and/or causes of aberrant regulation.

Results: This study identifies IH as the first non-malignant neoplasm expressing ectopic BORIS, an oncogene with expression normally limited to adult testes.. The paradoxically benign nature of IH despite BORIS expression could be explained by a 13 fold increase in CTCF, BORIS' only known antagonist, from proliferating to involuting IH. Interestingly, both proteins bind within IGF2 and H19. In the IH samples, as CTCF levels rose compared to BORIS, the IGF2 transcript decreased 6 fold. CTCF and BORIS likely regulate IGF2 by altering methylation of the region: The difference between CTCF and BORIS is most predictive of methylation levels at several imprinted sequences (R^2 = .9.) Throughout the 130 KB regulatory region controlling IGF2 and H19, BORIS favored methylation specific activation of IGF2 and repression of H19, while CTCF favored the converse. The degree of these effects strongly correlated with a common C/T polymorphisn at the IGF2 imprinting control region. Here the T allele was strikingly more sensitive to CTCF and BORIS than the C allele. Hence, the C/T polymorphism may be an important disease modifier of IH.

Conclusion: Identifying the aberrant expression of a known oncogene, BORIS in IH, suggests one factor driving early proliferation. Furthermore, the steady increase in the production of BORIS' antagonist, CTCF, may support the involutionary process. The interplay between these two proteins likely takes place at the level of DNA imprinting, as the difference between CTCF and BORIS was highly predictive of methylation levels within key regulatory regions of the IGF2/H19 locus, a region previously demonstrated to control the relative expression of both genes. A potentially important disease modifier may be the C/T polymorphism within CTCF binding site six, which strongly affects methylation of the region relative to the CTCF – BORIS difference. As 400,000 children are born each year in the US with IH, a clinical blood test resulting from this finding may be highly useful in predicting eventual tumor size and time to involution.

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STATEMENT OF PURPOSE

The purpose of this research project is to investigate the epi-genetic regulation of Insulin Like Growth Factor 2 (IGF2) and it's reciprocally imprinted transcript H19 in infantile hemangiomas. Through micro array studies and confirmatory RT PCR two independent labs have demonstrated that Insulin Like Growth Factor 2 (IGF2) levels decrease seven-fold form proliferative to involuting hemangiomas. There is substantial documentation that methylation abnormalities within the IGF2 and neighboring H19 loci are related to the overproduction of IGF2 in many distinct tumor types. Furthermore, Beckwith Wiedemann Syndrome (BWS) a disease of prenatal overgrowth--where visceral and coetaneous hemangiomas figure prominently--is caused by duplications or a loss of imprinting of the l1p15.5 locus, which contains IGF2. An investigation of the methylation status of this region may explain pathologic IGF2 overproduction in hemangiomas.

The Purpose of this thesis is to provide a potential mechanistic explanation of the disregulation of IGF2 in infantile hemangiomas via epi-genetic analysis of its imprinted locus. Through RT PCR and analysis of micro RNA transcripts, the potential consequences and causes of epi-genetic abnormalities found at IGF2/H19 specific to infantile hemangioma are explored.

Lastly, the question of infantile hemangiomas is framed within the broader scope of human imprinting by suggesting that these lesions could be an excellent and until now untapped resource to study human imprinting *in vivo*.

INTRODUCTION

Infantile hemangioma (IH) is the most common tumor of the pediatric age group, affecting up to 4% of newborns with 60% prevalence of the head or neck [1-3]. They are highly vascular lesions expressing markers most similar to that of placental tissue [1-7]. IHs range from inconsequential blemishes, to highly aggressive tumors that can threaten airways, sensory-neural structures, and potentially even high output cardiac failure secondary to tumor demand. However, belying this varied clinical picture is a consistent life history. For the first year, hemangiomas are highly active demonstrating initially, a histologic picture and behavior suggestive of malignancy: immature vascular channels, high mitotic indices, and strong positivity for proliferative markers such as Ki-67 in CD-31 positive (endothelial specific) Glut-1 positive (hemangioma specific) cells [3, 6, 7]. Despite these ominous beginnings, the most surprising aspect of IH is that they remain benign [2, 3]. Instead, the growth velocity slowly reverses leading to a "Quiescent Phase" of non growth (1 to 12 years) then transitioning into a regressive or "Involuting Phase" replacing once proliferative endothelium with now fibro-fatty residuum.

Despite the high prevalence of IH, little is known regarding the pathogenesis of the disease. However, a tumorigenic molecule, Insulin Like Growth Factor 2 (IGF2), has been implicated by microarray and confirmatory Real-Time PCR studies [8, 9]. IGF2 levels decrease over seven fold from proliferative to involuting IH. Furthermore, Beckwith-Wiedmann Syndrome (BWS) a disease of prenatal overgrowth--where visceral and cutaneous hemangiomas figure prominently--is caused by IGF2 overproduction via duplications or a

loss of imprinting of the 11p15.5 locus, which contains the IGF2 gene [10]. Furthermore, explant hemangioma cultures respond strongly to exogenous dosing of IGF2 [8].

Indeed, as suggested above, IGF2 itself is an imprinted gene. By definition, genes under the control of imprinting are expressed exclusively from one parentally contributed chromosome [11, 12]. Loss of imprinting (LOI) refers to the state of biallelic expression from a normally imprinted gene. Imprinted genes are discretely grouped within chromosomal structures and are often co-regulated, either positively or in a reciprocal fashion [13, 14]. Concerted inter-genic and intra-genic regulation of an imprinted gene cluster is accomplished through multiple chemical alterations of DNA and histones, i.e. the addition of methyl carbons to cytosines preceding guanines (otherwise known as DNA methylation) and the acetylation/methylation of histones [15-17]. These so called epi-genetic marks modify the array of DNA binding proteins capable of interacting with local chromatin structures. This in turn leads to changes in the three dimensional architecture of imprinted chromatin, granting differential access of promoters to enhancer elements [18, 19]. In addition, methylation alters the level of transcriptional factors bound to promoters [20]. The functional consequences of parental specific, epi-genetic marking is activation of a given parental allele with reciprocal silencing of the other allele [21].

In fact the IGF2/H19 locus now serves as a model of the phenomenon. The human IGF2 and H19 are adjacent genes approximately 130KB apart from one another and share common distal enhancers. Flanked by these two genes are seven potential “Cytosine preceding Guanine” (CpG) islands that are differentially methylated, with the paternal allele being generally more methylated than the maternal [22-25]. Embedded within each one of these

seven potential imprinting control regions (ICR's) is a binding site for the chromatin organizing protein CTCF (CCCTC-binding factor) [26]. CTCF binding to DNA is methylation sensitive [27-31]. Here CTCF binds only unmethylated chromatin—in this case maternally contributed DNA—and blocks access of IGF2 to its distal enhancers [17]. This so called insulator function eliminates IGF2 expression in favor of H19 upregulation on the unmethylated maternal chromosome. Conversely, paternally contributed, i.e. methylated, DNA resists CTCF binding and IGF2 is exclusively upregulated by said distal enhancers [32]. (See background figure 1, immediately following the introduction section) Although, any or all of these CTCF binding sites could potentially serve as an imprinting center, only CTCF binding site six (CTCF BS6) exhibits allele specific differential methylation making it the *de facto* imprinting control region (ICR) [33]. This imprinting control center not only regulates IGF2/H19 expression, but also maintains its imprinted status by protecting the region from inappropriate *de novo* methylation. [34-38]. Functional tests of these observations reveal that mutations within the ICR abrogate CTCF binding, allowing hypermethylation of the region and biallelic expression of IGF2 [19]. The importance of this region is also highlighted in common human diseases. Derangement of allele specific methylation of the ICR leads to LOI in colon cancer [39, 40], bladder cancer [26] and Wilms Tumor [41]. Furthermore, it is the specific hypermethylation of this region that leads to Beckwith-Wiedmann Syndrome a syndrome of hemi-hypertrophy associated with IGF2 overproduction [10]. On the contrary, hypomethylation of this region is associated with Russell-Silver Dwarfism: hemi-atrophy and IGF2 underproduction [42]. However, the strict 50% methylation rule of the H19 ICR was clearly established most strongly in the mouse [17]; most human studies have utilized either a *Sma1* methylation sensitive polymorphism in the nearby region of the H19 promoter as a surrogate for ICR

methylation or semi quantitative methylation sensitive PCR of the region proper [10, 23, 26, 39, 41]. Although these results did not dispute the mouse data, only recently has highly accurate methylation sensitive PCR with quantitative pyrosequencing been available for fully quantitative, high resolution, examination of CTCF binding site six [33]. Recent work demonstrates that there are actually three normal methylation states of the human ICR. The so-called Low (30%,) medium (50%) and high (70%) states of methylation were transmitted, as examined by pedigree analysis, in a fashion consistent with a one gene and three allele hypothesis [33]. However, this gene/allele was not identified. In addition, two groups have demonstrated a common C/T polymorphism at CpG number five in the core CTCF binding site of the ICR (CTCF BS 6) that blocks methylation at a specific cytosine [33, 43]. The functional consequences of these findings regarding IGF2 and H19 expression have yet to be determined.

To further complicate matters, as previously alluded, the ICR is not the only imprinted region in H19/IGF2. The H19 promoter, 40kb telomeric from the ICR is, a putative methylation sensitive repressor of H19, while regions within the IGF2 gene itself are hemi-methylated in a parental specific fashion [17, 39, 44]. Differentially methylated region zero or DMR0, in intron two of IGF2, is a maternally methylated inhibitor of IGF2 expression; loss of methylation here leads to LOI of IGF2 regardless of ICR status[39]. Also, multiple regions in exon 9 are paternally methylated and serve as methylation specific activators of IGF2 [17, 39, 44]. It was originally proposed that higher order chromatin conformations allow physical contact between these distant imprinted sequences, facilitating multi-regional coordination of IGF2 and H19 gene expression [15]. As CTCF BS 6 is the master control switch between H19 and IGF2, fine regulation is

delegated to DMR0, Exon 9 and the H19 promoter [15, 17]. Recently, this hypothesis was validated for the maternal mouse chromosome demonstrating that CTCF binds both the ICR, Exon 9 and the mouse equivalent of DMR0 forcing physical contact between all three regions [17]. This contact was necessary and sufficient to form a chromatin loop that excluded IGF2 from its distal promoters.

If allele specific methylation of imprinted genes composes a blueprint of differential gene regulation, the DNA binding protein CTCF has emerged from multiple lines of evidence as the reader of these blueprints. CTCF is otherwise known as the Regulator of Imprinted Sites or "RIS" secondary to its unique ability to partition DNA into active and inactive regions by insulating genes from proximate enhancers [45, 46]. It is the first discovered multivalent DNA binding protein with a total of 11 zinc fingers [47, 48]. By combinatorial interaction of these fingers, this protein is capable of ubiquitously binding unrelated sequences throughout the genome [47, 48]. The variability of its consensus binding sequences can only be matched by its multitude of recognized functions. Indeed, CTCF function is critical for normal cellular processes, as it is highly conserved from *xenopus*, to humans [49-52]. Furthermore, it's demonstrated functions range from X inactivation to genome wide regulation [53, 54]. However, among the sundry functions of this versatile protein, imprinting maintenance appears to be the most consistent. Strikingly, all imprinted genes identified thus far contain insulating boundaries that bind CTCF [46, 48, 55, 56]. Ultimately, CTCF is a growth arresting gene: transfection of CTCF into a multiplicity of cell lines did not induce apoptosis, but rather profound growth arrest, freezing cells before the S phase [57]. Conversely, partial loss of function of CTCF is associated with malignancy: CTCF maps to the smallest region of overlap observed at

16q22 common to breast, prostate, and Wilms tumor [18, 19, 58]. Also, tumor specific missense mutations within the zinc finger binding domain of CTCF are reported in those same forms of cancer [19]. Interestingly, these mutations did not completely nullify CTCF binding to DNA, but rather selective binding to important growth regulating genes such as IGF2, c-Myc, BRCA 1, ARF, PLK and PIM1; however, other growth neutral genes containing CTCF sensitive insulator sites, such as the beta-globin gene and APP promoter, remained unaffected [19]. These data suggest that single mutations within the ZF domain of CTCF can dramatically change its DNA binding spectrum, thus altering the genome wide transcriptome at a fundamental level.

Despite the apparently unique role for CTCF and its near singular structure, a protein that shares the same 11 zinc finger binding domains as CTCF was recently cloned [59]. Presumably, this new protein can bind to the same DNA sequences as CTCF suggesting that they may compete for the same sites. As CTCF's moniker is the "Regulator of Imprinted Sites" (RIS,) this protein was dubbed the Brother of Regulator of Imprinted Sites or "BORIS." Indeed, if these two proteins are siblings, they represent the Cain and Abel of chromatin—similar but opposing functions. As loss of heterozygosity of CTCF at 16q22 is strongly associated with a cadre of malignancies, amplification of the chromosomal region containing BORIS, 20q13, is commonly associated with the same grouping of cancers [60-65]. This led to the suggestion that 20q13 contains a common oncogene [47]. In terms of normal expression, CTCF is nearly ubiquitous, while BORIS is confined to the one tissue that CTCF is not expressed, adult testes [59]. Furthermore, testes specific expression of BORIS is confined to the subset of CTCF negative cells where methylation imprints are removed and paternally reestablished [59]. The function

of BORIS is so specialized that ectopic expression, that is transcription outside of the testes, is invariably associated with cancer [47, 59]. Given these data, the question still remained, what are the effects of BORIS on the H19/IGF2 locus specifically. Recent work demonstrates that in *xenopus* oocytes, the co-injection of BORIS along with methylation cofactors (DNA methyl transferases 3a, b, or l, and the histone methylating enzyme PRMT7), is followed by increased methylation at IGF2/H19 ICR [66]. This is the first *in vitro* experiment directly testing the hypothesis that increased expression of BORIS leads to increased methylation at an important imprinting control center. Mechanistically speaking, BORIS physically associates with PRMT7; this interaction was followed by histone methylation at the IGF2/H19 ICR, which was then followed by direct DNA methylation. One explanation for these findings is that BORIS physically guides histone methylating enzymes to the ICR and histone methylation serves as a mark for the DNA methyl transferases to directly methylate DNA. It is important to bear in mind that, although not directly tested in these experiments, increased levels of methylation at the IGF2/H19 ICR is associated with upregulation of the IGF2 transcript. Moreover, upregulation of IGF2 by loss of imprinting is commonly associated with ectopic BORIS expression in cancer [67]. In short, BORIS may upregulate IGF2 while CTCF is known to represses it.

Although chromatin architecture is pivotal to gene regulation--CTCF, if not also BORIS, is central to this process--it is important to keep in mind that only 4% of all transcribed genes are known to be translated [68-70]. The remaining 96% of the transcriptome remains as potentially functional RNA. In fact, recent micro array studies using chips designed to detect transcripts from the whole gemone rather than just exons of known

proteins, found that almost 50% of the human genome is transcribed [70]. This topic is particularly germane to the IGF2/H19 region as H19 itself is a non translated RNA. The precise functions of H19 remain to be elucidated; however, knockout and transfection experiments have offered clues. Although H19-/- mice were viable, they were often 25% larger than their H19+/+ littermates [71, 72]. Several theories, ranging from the pedestrian to the exotic, have been evoked to explain this phenomenon. An example of the former is that the knockouts themselves disrupted local chromatin structure, thus relaxing IGF2 imprinting [71, 72]. An example of the latter is that, H19 represses IGF2 in a fashion similar to the functional RNA "xist" inactivating the X chromosome. [73-75]. Although none of these theories are conclusive, there is some evidence that the H19 transcript itself modulates IGF2. In fact, H19 RNA was found to be associated with polysomes, possibly affecting IGF2 at the translational level [76]. Second, in vitro experiments showed that H19 RNA down regulated IGF2 transcription specifically from its third promoter [77]. Third, H19 associates with IGF2 MRNA binding protein 1 (IMP1), a known upregulator of IGF2 translation [76]. Lastly, and perhaps most promisingly, recent work confirms that H19 is the parent transcript for the conserved microRNA-675 [78].

Micro RNAs are a class of small non-coding transcripts that, in their mature form, are 18-25 base pairs long. These genes are often arranged in tightly packed clusters under common promoters [79] (See background Figures 2A and 2B, immediately following the introduction section.) Despite their size, these genes exert powerful influences on the transcriptome in a two stage process: 1)The micro-RNA binds to larger transcripts in a target specific manner via complimentarity (Usually in the 3' UTR.) 2)This binding creates localized double stranded RNA which targets the mRNA for destruction or

interferes with translation [80-83] (See backgroung figure 2A.) Micro RNA expression is often downregulated in cancer, and common chromosomal breakpoints associated with malignancy bisect micro-RNA clusters [79]. For example, lymphocytic lymphoma is strongly associated with a chromosome 13q14 deletion [79]. Given that the largest open reading frame (ORF) within this deletion, is too small to code for a protein, the most likely gene of interest is the micro RNA cluster mir-15a–16. On the other hand, select micro RNAs are mapped to common sites of chromosomal amplifications in malignancy. The chromosome 13q31 amplification, commonly associated with both lymphomas and solid tumors, contains the microRNA-17-92 cluster [84]. Again, this gene has a very small ORF likely insufficient for translation. Yet, elevated expression of the mature micro RNAs from this cluster, has been verified in primary lymphoma as well as a wide range of tumor-derived cell lines [85, 86]. Furthermore, enforced expression of the 17-92 cluster in a mouse B-Cell Lymphoma model, significantly accelerated tumor development [85, 86]**.** Intriguingly, many of these micro-RNA clusters are arranged within imprinted genes that contain CTCF/BORIS binding sites [87]. Moreover, the micro RNA clusters in the imprinted DLK/RTL1 locus are also subject to imprinting, at least in the mouse [88]. This is the first direct evidence that micro RNAs are actually imprinted rather than just clustered within imprinting control centers. Imprinted micro RNAs are of particular interest because, many of them could be regulated, at least partially, by CTCF and BORIS, providing another link between micro RNA expression, target gene regulation, and imprinting. Moreover, through prediction algorithms, a set of two related micro-RNAs (mir23a and mir23b) that potentially target both CTCF and BORIS have been identified. The implication being, not only can imprinting affect micro RNAs, but micro RNAs may also affect imprinting. To date, mir-23a and 23b, are

differentially regulated in cardiac hypertrophy as well as leiomyoma generation[4, 89], one wonders if it may be through manipulation of imprinted genes via modulation of CTCF or BORIS.

Summary of Findings

This study confirmed that IGF2 RNA expression decreases six fold from proliferating to involuting IH. Interestingly, hemangioma tissue expresses highly significant levels of BORIS, as the endothelial cell control lines were BORIS negative. This places hemangiomas within the unique category of being a BORIS positive tumor that is non-malignant. This unusual finding may be explained by a concomitant 13 fold increase in CTCF from the proliferating to involuting samples. Not surprisingly, the difference between CTCF and BORIS in a given tissue is highly predictive of IGF2 expression: Roughly equal levels of CTCF and BORIS are produced in proliferative lesions while CTCF is highly favored by the time of involution. These two factors may modulate IGF2 expression by interacting with this gene's imprinted regions: higher relative levels of BORIS were consistent with further methylation of both the IGF2/H19 ICR (CTCF BS 6) and Exon9, both methylation sensitive activators. Conversely, higher levels of CTCF correlated strongly with hypermethylation of the methylation sensitive repressor DMR0. Within the core binding site of the IGF2/H19 ICR, methylation analysis identified a previously catalogued C/T polymorphism that appears to change the sensitivity of both IGF2 production and Exon 9 methylation to the relative amounts of CTCF and BORIS. This evidence 1) supports the conclusion that the IGF2/H19 ICR and Exon 9 interact,

despite the fact that they are approximately 80KB apart and 2) suggests a possible mechanism, modulated by a common polymorphism in a critical binding site, of the variable clinical behavior underlying these lesions. CTCF and BORIS appear to strongly regulate IGF2 production, however, the mechanisms regulating CTCF and BORIS themselves remain unknown. Through a search of the three major micro RNA target prediction programs available, one set of closely related micro RNA's consistently targeted both CTCF and BORIS. By correlating micro RNA 23a (mir-23a) and mir-23b to CTCF and BORIS levels in the IH samples tested, mir-23a correlated with CTCF and BORIS down regulation equally well, yet mir-23b only correlated with CTCF down regulation. This finding was bolstered by the fact that mir23b could theoretically form an extra bond with CTCF over BORIS**.** In addition, the difference between mir23a and mir23b strongly correlated with the difference between CTCF and BORIS. Mir23b may be a potential anti-target of BORIS relative to CTCF: thus increased mir23b compared to mir23a expression would effectively lead to increased levels of BORIS over CTCF. These data suggest a link between differential expression of two micro RNAs known to be disregulated in hypertrophic tissue (mir-23a and 23b), the relative levels of two opposing chromatin organizing proteins (CTCF and BORIS), and the regulation of an imprinted gene known to promote growth (IGF2.)

METHODS AND MATERIALS

Specimen Collection

(Performed by the Student)

Twenty-one hemangioma samples subjected to methylation analysis, nine samples were found to have suitably intact RNA for quantitative RT PCR with 18s to 28s ratios equal or greater than 1.8, and eight samples were analyzed by Western blotting. All samples were collected in accordance with an approved HIC protocol (#0507000430) as reviewed by the Yale University Medical School IRB. As these samples were collected from children, fully informed parental, and childhood assent when age appropriate, was obtained prior to surgery. Only the tissue remaining--following collection of the pathological specimen--was used for this experiment. Those specimens later confirmed to be hemangioma tissue, as assessed by Glut-1 positivity, were considered for this project. Specimens for transcriptional analysis were separated into three categories: 1) Proliferative, 2) Quiescent, and 3) Involuting phases. These categories were determined on a clinical basis, as well as by age. Proliferative hemangiomas: Less than 1.5 years of age with interval growth between the last two clinic visits preceding surgery. Quiescent hemangiomas: Older than 1 year demonstrating no interval growth between the last two clinic visits preceding surgery. Involuting hemangiomas: At least two years old with interval regression by measurement between the last two clinic visits preceding surgery.

DNA Preservation and Extraction

(Performed by the Student)

Immediately following tissue resection, at least 100mg of tissue was frozen from each sample in a 10ml Falcon tube on dry ice for later processing. DNA was isolated using the Qiagen DNeasy Tissue Mini Kit according to the manufacturer's protocol with the following exceptions. 50mg of tissue were used if the tissue consisted of fibro-fatty components, otherwise the recommended 25mg of tissue was utilized. Before tissue lysis, samples were liquefied using straight razor blades as opposed to Quiashredder columns for fibrous tissue as to decrease the possibility of DNA shearing. Furthermore, all vortexing steps were minimized for the same purpose. This is necessary to preserve DNA integrity for bisulfite conversion as this process destroys as much as 90% of the starting material [90]. Lastly, the tissue was subjected to lysis with buffer ATL and proteinase K digestion overnight as opposed to the recommended 1-3 hours. Samples were eluted in buffer AE and QC tested via spectrophotometry, and gel electrophoresis on 2% agarose stained with ethidium bromide. Only samples with an A260/A280 measurement of 1.8 or above that ran as a single band on the gel were further analyzed.

RNA Preservation and Extraction

(Performed by the Student)

Immediately following tissue resection, 100-500mg of tissue was minced in 10ml of Quiagen RNA Later solution with straight razors into pieces no larger than 1mm in any dimension. Samples were stored in 50ml Falcon tubes with an additional 10ml of RNA later solution. Samples were then stored at -20 degrees C. overnight and then frozen at -80 degrees C until

such time as RNA Extraction could be completed. RNA was extracted by first allowing the RNA later solution to thaw. This was followed by straining of the sample and immediate liquid nitrogen powder homogenization in a mortar and pestle. All implements were baked at 200 degrees C. overnight to eradicate RNAase enzymes. Following homogenization, RNA was extracted using Invitrogen Trizol reagent according to manufacturer's specifications with the following exceptions. Once the initial phase separation was accomplished with the addition of phenol/chloroform, the samples were vigorously vortexed to shear genomic DNA. This helps insure that the DNA will migrate completely into the organic phase instead of remaining at the inter-phase, which may contaminate the RNA sample. Following the phenol/chloroform extraction, the supernatant (aqueous RNA phase) was subjected again to a 1/24 Iso-amyl-alcohol/chloroform extraction to minimize potential phenol contamination, which could inhibit downstream enzymatic applications. After the iso-propyl alcohol precipitation and ethanol washing steps, the pellets were allowed to dry for 15 to 30 minutes and resuspended in nuclease free water and stored at -80 C. To remove potential genomic contamination, 10 μg of total RNA from each sample was then treated with DNase Qiagen mini-elute columns according to manufacturer's specifications. RNA integrity was then assessed using 1μl of sample on the Agilent bioanalyzer 2100 (provided as a service of the Keck Center at Yale University.) Band intensities of 18s and 28s RNA were quantitated and samples with an 18s/28s ratio of 1.8 or greater were utilized for quantitative RT PCR. Following QC each sample was converted into cDNA using the ABI 4368813 cDNA archive kit. All samples were then stored at -80 degrees C.

Endothelial Cell Purification and Culturing

(Performed by the Student)

This protocol was optimized at the Yale Skin Diseases Research Center, New Haven CT. Human dermal microvascular endothelial cells (HDMEC) were isolated from normal adult skin obtained as discarded tissue from Yale-New Haven Hospital, New Haven, CT, under an approved HIC protocol. Roughly 3cm by 10cm sections of skin are stretched flat and planed using a Webster skin graft knife set to .016 inch depth. Planed skin is incubated at room temperature in dispase (Collaborative Biomedical Products.) The epidermis can then be peeled away. The remaining dermis is then minced into .5 cm^2 pieces and passed through a 70μM metal mesh. The dispase is then neutralized by the addition of two volumes of FBS. Suspended cells are then spun at 1000g for 5 minutes and the supernatant is removed. Cells are then suspended in 5ml of fully supplemented defined EGM2 media and plated on a single well of a fibronectin (40μg/ml) coated plate. The primary culture is allowed to incubate overnight at 37 degrees C in 5% $C0_2$. The media is replaced on the following day, with replenishments every 2 days until the primary culture is confluent. Cells are then trypsinized and subjected to endothelial cell selection using a mouse IGG anti-CD31 antibody (Dako) conjugated to MACSiMAG magnetic beads and passed through a micropore column placed in a magnetic field (miniMACS Separator, Myltenyi Biotech) according to the manufacturer's protocol. Purified endothelial cells are then plated on fibronectin coated plastic and grown to confluence, then expanded. Cells at passage 3 are then subjected to FACS analysis, cultures that are 90% CD31 positive or greater were then DNA and RNA extracted**.** Human Umbilical Vein Endothelial Cells (HUVEC) were isolated as a service of the Yale Skin Diseases Research Center and pooled from three separate donor cords, using a similar protocol. Neonatal HDMEC cells, isolated from pooled foreskin (n=3), were

purchased from Cambrex and grown to confluence as noted above. Cells were RNA/DNA extracted at P4.

Quantitative PCR for CTCF, BORIS, H19 and IGF2

(Performed as a service by the Keck Center at Yale University)

9 hemangioma samples (3 proliferative, 3 quiescent, 3 involuting) and 5 endothelial cell control lines were RNA extracted as previously specified and subjected to fluorescent quantitative RT-PCR using ABI Taqman primers that were previously validated by the manufacturer. All primers span intron exon boundaries, further eliminating the possibility of false signals due to genomic contamination. The assays were: IGF2--assay number Hs00171254_m1, H19—assay number Hs00399294_g1, CTCF—assay number Hs00198081_m1, and BORIS—assay number Hs00540744_m1. Gene quantification was performed using the standard curve method: For each gene, a pooled sample of cDNA (equal contributions from each sample) was used in successive two fold dilutions, beginning from 50 ng and ending with .39 ng, to correlate a CT value (cycle number required to reach the threshold detection of PCR product) with absolute quantity of RNA starting material. Each reaction was performed in duplicate with four empty wells as negative controls. CT values from unknown samples can then be correlated with the absolute quantity of RNA in ng present in the reaction vessel. This number is normalized to the absolute quantity of GAPDH control RNA present. The standard curve method allows absolute quantities of RNA to be determined making it possible to compare one transcript directly to another. 50 ng of RNA were used for each unknown sample in 20μl reactions using 1μl of 20x target assay mix (Primers) and 10 μl of 2x Taqman PCR master mix on a 384 well plate with

optical plate cover. All reactions were performed on the ABI 79005 thermocycler using default cycling conditions previously optimized for these assays. Reactions were performed in duplicate and average CT values, if they agreed within 0.4 cycles, were used to calculate absolute quantity.

Western Analysis

(Performed by the Student)

8 samples were subjected to Western analysis. Briefly: 50 mg of each sample were mechanically homogenized with a rotary homogenizer in 200ml of RIPA lysis buffer. The homogenates were spun at 15,000g for 10 minutes and protein concentrations of the supernatant were determined using the Bio-Rad Protein Assay with subsequent optical density testing according to manufacturers specifications. Lysates were made using a standard beta-mercapto-ethanol with SDS buffer at a concentration of 4 μg per μl and heat treated at 95 degrees C for five minutes. PAGE was performed with 36μg of protein per well in NuPage 10% Bis-Tris precast gels in MOPS buffer at 100 volts. PAGE separated proteins were then transferred for two hours to a PVDF membrane (Bio-Rad) in a standard transfer buffer at 100mAmps. The membrane was blocked in TBST with 5% cows milk for one hour and probed with anti BORIS antibody (Abcam #ab18337) 1/5000 dilution in TBST with 5% cows milk overnight. Membranes were washed in TBST for one half of an hour and probed with anti-rabbit secondary antibody conjugated to horseradish peroxidase. Membranes were washed for one half of an hour in TBST and then visualized with ECL and photographic film. The membrane was then probed with anti CTCF antibody (Abcam #10571) overnight, washed and then visualized as noted above. As anti-CTCF and anti-BORIS were both rabbit polyclonal antibodies they could be visualized simultaneously on the same film following

incubation with a sheep anti-rabbit secondary antibody conjugated to horseradish peroxidase (Chemicon AP304P) and ECL treatment. Images were then scanned and adjusted for brightness and contrast in Adobe Photoshop.

Bisulfite Methylation Analysis Using Conventional Sequencing

(Performed by the Student)

Bisulfite treatment efficiently converts unmethylated cytosines into uracils, while 5-methyl-cytosines remain intact. This conversion locks methylated and unmethylated cytosines into C/T polymorphisms respectively. Following bisulfite specific PCR and sequencing, the relative contributions of cytosine to thymine signals at this newly created polymorphic site is proportionate to the level of methylated DNA at the nucleotide position in question (See methods Fig. 1, immediately following the methods and materials section.) Conventional sequencing allows for a semi-quantitative visual comparison between samples.

Bisulfite Conversion: Briefly: 2µl of DNA suspended in 50µl of TE was first denatured by adding 5µl of freshly prepared NaOH (3 M, final concentration 0.3 M) and incubated at 37-42°C for 15-30 min. The denatured DNA was then combined with 1)510µl of freshly prepared 40.5% sodium bisulfite by weight, 2)30µl 10mM hydroquinone, and water up to a volume of 610µl. The reactions were covered with mineral oil and placed in a 55° C water bath for 8-16 hours. DNA was then purified using DNA binding resin with vacuum column filter binding (Promega, DNA Wizard Cleanup Kit) according to the manufacturer's specifications and eluted in 50µl of TE. The samples were then denatured with NaOH as above and left to incubate at RT for 15 minutes. The pH was

then neutralized by adding 3M ammonium acetate. DNA was then ethanol precipitated using a glycogen carrier/visualizer and resuspended in 20µl of H_20 and immediately stored at -40° C.

PCR: The proximal region of IGF2 Exon 9 was amplified by a previously described primer pair specific for bisulfite converted DNA [44].

F: 5-GTAGGGGTTTGTTTGTTTTTTTG-3; R: 5- CTACTATACTTCCTCAACCC-3

50µl reactions containing: 5µl 10x PCR buffer (Invitrogen: Platinum Taq), 1µl DNTP, 1.5µl 50mm $MgCl_2$ .5µl of combined forward and reverse 25pm primers, 41.8µl H_2O, and .2µl Platinum Taq (Invitrogen).

40 cycles of PCR were performed with the following conditions:

95° - 4 minutes

95° - 30 seconds

48° - 30 seconds

72° - 30 seconds

72° - 5 minutes

DMR0 consists of three CpG's in close proximity witin intron 2. This region was amplified by a previously described bisulfite specific primer pair [44].

F: 5-GTTAAGGTAGTTTTTTTGGG-3; R: 5-AATTAACCCACCTTAAAAAATC-3

50µl reactions containing: 5µl 10x PCR buffer (Invitrogen for Platinum Taq), 1µl DNTP, 1.5µl 50mm $MgCl_2$ .5µl of combined forward and reverse 25pm primers, 41.8µl H_2O, and .2µl Platinum Taq (Invitrogen).

35 cycles of PCR were performed with the following conditions:

95° - 4 minutes

95° - 30 seconds

52° - 30 seconds

72° - 30 seconds

72° - 5 minutes

PCR products were gel purified on 2% agarose, using a Qiagen Gel Extraction kit according to the manufacturer's specifications. The forward primer was used to sequence the PCR products (Keck Center at Yale University) and visual comparisons between samples were performed using the Four Peaks version 1.7 sequence viewer (Mekentosj, Netherlands.)

Bisulfite Methylation Analysis Using Quantitative Pyrosequencing

(Performed as a Service by Epi-Gen Dx)

First described by DuPont *et al* [91], quantitative bisulfate pyrosequencing for CpG islands (Pyro Q-CpG) is a real-time sequencing-based DNA methylation analysis that quantifies methylation patterns of consecutive CpG sites individually.

Briefly 1000 ng of sample DNA was bisulfate treated using the Zymo DNA Methylation Kit (Zymo research, Orange, CA) according to the manufacturers instructions. Bisulfate treated DNA is eluted in10 ul of nuclease free water, 1 ul of eluate is used for each PCR reaction. PCR was performed using 10X PCR buffer, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.2 μM each of forward and reverse primers, HotStar DNA polymerase (Qiagen Inc.) 1.25 U, and 100 ng of bisulfite treated DNA per 50 μl reaction. PCR cycling conditions were: 94ºC for15 min; then 45 cycles at 94ºC 30 s; 58ºC 30 s; 72ºC 30 s; 72ºC 5 min. The products were than held at 4°C. Each PCR was performed with one biotinylated primer, allowing sense and antisense strands to be separated. Single-stranded DNA templates were pyrosequenced according to the manufacture's protocol (Biotage, Kungsgatan, Sweden). The target CpG-islands and the primer sequences are shown in supporting text below. Pyrosequencing reactions are subject to bias: 1)bisulfite conversion can be incomplete and 2)Given PCR primers may preferentially amplify the methylated alleles. These concerns were addressed by the following: 1)Amplicons were designed such that they contained at least 1 unmethylated cytosine, that is a cytosine that does not preceede a guanine. Completed bisulfite reactions lead to the complete conversion of this cysosine into a thymidine. Only those reactions that demonstrated

complete conversion were analyzed. 2)Primer bias was controlled for by establishing methylation curves of 100% methylated DNA titrated against known amounts of whole genome amplified PCR products that, by definition, are unmethylated. These methylation curves allow experimental samples to be calibrated against known standards.

H19 CTCF Binding Site 6:

Primers:

The reverse PCR primer is biotin labeled on the 5' end

SNP ID	Type of primer	Primer sequence 5'->3'	Length
DMR H19 CTCF Binding Sites	PCR F	TGGGTATTTTTGGAGGTTTTTTT	23
DMR H19 CTCF Binding Sites	PCR R Biotin	TCCCATAAATATCCTATTCCCAAA	24
DMR H19 CTCF Binding Sites #22 - #27	Seq F	TTTATYGTTTGGATGG	16
DMR H19 CTCF Binding Sites #28 - #33	Seq F	GTAGGTTTATATATTATAG	19
DMR H19 CTCF Binding Sites #34 - #37	Seq F	GTTTYGGGTTATTTAAGTTA	20

Original sequence

TGGGTATTTCTGGAGGCTTCTCCTTCGGTCTCACCGCCTGGATGGCA
CGGAATTGGTTGTAGTTGTGGAATCGGAAGTGGCCGCGCGGCGGCAGT
GCAGGCTCACACATCACAGCCCGAGCCCGCCCCAACTGGGGTTCGCCC
GTGGAAACGTCCCGGGTCACCCAAGCCACGCGTCGCAGGGTTCACGG
GGGTCA**TCTGGGAATAGGACACTCATAGGA**

Bisulfite converted PCR amplicon sequence:

TGGGTATTTTTGGAGGTTTTTTTTTYGGT**TTTATYGTTTGGATGG**TA
YGGAATTGGTTGTAGTTGTGGAATYGGAAGTGGTYGYGYGGYGGTAG
T**GTAGGTTTATATATTATAG**TTYGAGTTYGTTTTAATTGGGGTTYGTT
YGTGGAAAY**GTTTYGGGTTATTTAAGTTA**YGYGTYGTAGGGTTTAY
GGGGGTTA**TTTGGGAATAGGATATTTATAGGA**

Note: Letters in red are PCR primers. Underlined bold are Pyrosequencing primers. This PCR amplicon covers 16 CpG sites (from #22 to #37). There is a mismatch A at the reverse PCR primer highlighted in blue. The base highlighted in Orange was found to be polymorphic by Pyrosequencing. Yellow is the core CTCF binding site.

Human DMR IGF2 Intron 2:
Primers:

The reverse PCR primer is biotin labeled on the 5' end.

SNP ID	Type of primer	Primer sequence 5'->3'	Length
DMR IGF2 Intron 2	PCR F	GGGGGTTTATTTTTTTAGGAAG	22
DMR IGF2 Intron 2	PCR R Biotin	AAAACCACTAAACACACAACTCT	23
DMR IGF2 Intron 2 #13 - #15	Seq F	TTTATTTTTTAGGAAGTAT	20

Original sequence

CCCAGGGTGGTGTCTGTGGGGAGGGGGTTCATTTCCCCAGGAAGCACAGCCACGCCGTCCCTCACTGGCCTCGTCAAG**CAGAGCTGTGTGTCCAGT**

Bisulfite converted PCR amplicon sequence :

TTTAGGGTGGTGTTTGTGGGGAGGGGGTTTATT**TTTTTAGGAAGTATAGTTA**YGTYGTTTTTTATTGGTTTYGTTAAG**TAGAGTTGTGTGTTTAGT**

Note: Letters in red are PCR primers. Underlined bold are Pyrosequencing primers. This PCR amplicon covers 3 CpG, yellow(from #13 to #15).

Human DMR IGF2 Exon 9
Primers:

The reverse PCR primer is biotin labeled on the 5' end

SNP ID	Type of primer	Primer sequence 5'->3'	Length
DMR IGF2 Exon 9	PCR F	GGGTTTTGGG TGGGTAGAGT	20
DMR IGF2 Exon 9	PCR R Biotin	CCAAAACAAC TTCCCCAAAT	20
DMR IGF2 Exon 9 CpG sites	Seq F	GTTTGGTTTTT TTGAA	16

Original reverse complimentary sequence:

GGTCTTGGGTGGGTAGAGCAATCAGGGGACGGTGACGTTTGGCCTCCC
TGAACGCCTCGAGCTCCTTGGCGAGCACGTGACCCCGGCGGGCACGC
AGGAGGGCAGGCAGGCCCCTGCGCAGGCGCTGGGTGGACTGCTTCCA
GGTGTCATATTGGAAGAACTTGCCCACGGGGTATCTGGGGAAGTTGTC
CT

Bisulfite converted PCR amplicon sequence:

GGGTTTTGGGTGGGTAGAGTAATTAGGGGAYGGTGAY**GTTTGGTTT**
TTTTGAAYGTTTYGAGTTTTTTGGYGAGTAYGTGATTTYGGYGGGTAY
GTAGGAGGGTAGGTAGGTTTTTGYGTAGGYGTTGGGTGGATTGTTTTT
AGGTGTTATATTGGAAGAATTTGTTTAYGGGGT**ATTTGGGGAAGTTG**
TTTTGG

Note: Letters in red are PCR primers. Underlined bold are Pyrosequencing primers. This PCR amplicon covers 12 CpG sites yellow.

H19 Promoter

Primers:

H19Prom-FP2	5'-GGGAGGGTTTTGTTTTGATTG-3'
H19Prom-RP Biotin	5'-TTCCCCACTTCCCCAATTT-3'
H19PromFS3	5'-GTTATTTTAGTTAGAAAAAG-3'

Original Sequence:

GGGAGGGCCCTGCTCTGATTGGCCGGCAGGGCAGGGGCGGGAAT
TCTGGGCGGGGCCACCCCAGTTAGAAAAAGCCCGGGCTAGGACC
GAGGAGCAGGGTGAGGGAGGGGGTGGGATGGGTGGGG

Bisulfite Converted PCR Amplicon Sequence:

GGGAGGGTTTTGTTTTGATTGGTYGGTAGGGTAGGGGYGGGAATT
TTGGGYGGGGTTATTTTAGTTAGAAAAAGTTYGGGTTAGGATYGA
GGAGTAGGGTGAGGGAGGGGGTGGGATGGGTGGGG

Note: Letters in red are PCR primers. Underlined bold are Pyrosequencing primers. 2 CpG sites were quantified, yellow.

Genomic Southern Analysis for H19

(Performed as a Service by the Washington University Molecular Diagnostics Lab)

Ten micrograms of DNA were digested at 25°C overnight with 40 U of *Sma*I. This liberates a 1.8 KB fragment containing the H19 promoter region (corresponding to nucleotides 11803-13603 of the human *H19* probe (See methods Fig. 2, immediately following the methods and materials section.) The digestion was followed by an additional incubation at 37°C for 4 h with 40 U of *Pst*I. *Pst1* is a methylation sensitive restriction enzyme that cuts the 1.8 KB fragment of interest into a .3 and 1.5 KB fragment only if the DNA is unmethylated at the specific CCCGGG site of interest (number 806,386--genbank gi:51470970.). The digested DNA was electrophoresed on a 1% agarose gel, transferred to Hybond-N^+ (Amersham), and hybridized with the 1-kb *Pst*I + *Sma*I fragment isolated from an *H19* genomic clone that was previously radio-labeled with [^{32}P]dATP. Signals were quantified using a PhosphorImager (Molecular Dynamics). Percent methylation is calculated by dividing the 1.8KB band intensity by the sum of the intensities of both bands. Normal methylation was previously established by this lab as 55% +/- 7 (N = 50.) DNA derived from hemangioma samples (13) as well as patient matched control blood (13) were analyzed and compared.

Micro RNA Microarray

(Performed as a Service by LC Sciences)

This experiment consisted of five hemangioma micro-RNA samples ages: 95, 420, 547, 760 and 1520 days. The ages of samples were calculated from birth to time of resection.

In addition, two normal endothelial cell control lines were analyzed at passage 4: 1)HUVEC and HDMEC. Each probe was included on the chip seven times and from these signals an average and standard deviation were calculated. P-values of the t-test were calculated for any detected signal for one transcript between one sample and another, those with values less than 0.01 were considered significant and subjected to unbiased cluster analysis. Regression analysis was performed on those transcripts that were predicted to target CTCF or BORIS as calculated by the three primary micro-RNA target prediction programs.

The assay started with 2 to 5 µg of total RNA sample, which was size fractionated using a YM-100 Micro-con centrifugal filter (from Millipore.) The small RNAs (< 300 nt) isolated were 3'-extended with a poly(A)tail using poly(A) polymerase. An oligonucleotide tag was then ligated to the poly(A) tail for later fluorescent dye staining. Hybridization was performed overnight on a µParaflo microfluidic chip using a micro-circulation pump (Atactic Technologies). On the microfluidic chip, each detection probe consisted of a chemically modified nucleotide coding segment complementary to a target micro RNA (from MirBase 4.0, http://microrna.sanger.ac.uk/sequences/) and a spacer segment of polyethylene glycol to extend the coding segment away from the substrate. The detection probes were made by in situ synthesis using PGR (photo-generated reagent) chemistry. The hybridization melting temperatures were balanced by chemical modifications of the detection probes. 100 µL 6xSSPE buffer (0.90 M NaCl, 60 mMNa2HPO4, 6 mM EDTA, pH 6.8) containing 25% formamide at 34 °C was used for hybridization. Following hybridization, fluorescence labeling using tag-specific Cy3 dye followed. Hybridization images were collected using a laser scanner (GenePix 4000B,

Molecular Device) and digitized using Array-Proimage analysis software (Media Cybernetics). Data were analyzed by first subtracting the background and then normalizing the signals using a LOWESS filter (Locally-weighted Regression)10.

Statistical Analysis

(Performed by the Student)

Unless specified, all categorical analysis was performed using Microsoft Excel two tailed t-tests. All data is reported as a mean with standard deviation (quantitative RT PCR data is presented as a unit-less ratio of transcript expression divided by GAPDH expression, both originally measured in nanograms.) Correlation analysis utilized the least squares regression function of Microsoft Excel where the best fit curve and R^2 values are reported. Statistical analysis of the micro RNA microarray data is reported in the microarray methods section.

Summary of Methods:

Performed by the Student	**Performed by Service Providers**
Specimen Collection	Quantitative RT PCR
DNA Preservation and Extraction	Quantitative Pyrosequencing for Methylation Analysis
RNA Preservation and Extraction	Micro-RNA Microarray Analysis
cDNA Synthesis	Genomic Southern Analysis
Endothelial Cell Culture Purification	
Endothelial Cell Culture Maintenance	
FACS analysis of Endothelial Cell Culture	
Western Analysis of CTCF and BORIS	
Bisulfite Specific PCR for Methylation Analysis	
Data and Statistical Analysis	

RESULTS

IGF2 decreases six fold in involuting hemangiomas

By performing quantitative RT PCR on 9 hemangioma samples (3 proliferative, 3 quiescent and 3 involuting) and comparing these results to 5 normal endothelial cell control lines, abnormal expression of IGF2 in infantile hemangiomas was confirmed (See results figure 1, immediately following the references section.) All hemangioma tissues made IGF2 to varying degrees (Proliferative = 2.28 SD+/- 0.863, Quiescent = 3.866 SD+/- 1.19, and Involuting = .384 SD+/- .005.) [quantitative RT PCR data is presented as a unit-less ratio of transcript expression divided by GAPDH expression, both originally measured in nanograms.] The endothelial cell control lines, however, produced no IGF2. This indicates that IGF2 is not part of the normal transcriptome of endothelial cells as even neonatal endothelial cells (NHDMEC) and human umbilical vein endothelial cells (HUVEC), closest in age to hemangioma tissue, were IGF2 negative (P tissue vs. cell lines = <.00001.) Furthermore, these data confirm previous studies concluding that IGF2 RNA expression significantly drops from proliferating to involuting hemangiomas, six fold in this study p = .002 (See values listed above.) Curiously, the "quiescent" hemangiomas made highly variable levels of IGF2. To explain this variance, RT PCR of of CTCF and BORIS, proteins capable of binding to known regulatory sites within the imprinting control regions of IGF2, was conducted.

CTCF and BORIS are Co-expressed in IH at the Transcript and Protein Levels

IH of all categories produce both the BORIS transcript and protein (Figure 1B and 1D), while the transcript was undetectable in all five endothelial cell control lines (P = .038.) (EC = 0, proliferative = 1.01 SD+/- 0.56, quiescent = 8.5 SD+/- 3, involuting = 2.8 SD+/- 0.17.) Furthermore, the levels of BORIS are even more striking when compared to the respective expression of CTCF: only the involuted hemangiomas produced relatively less BORIS than CTCF. It is intriguing to note that IGF2 transcript expression appears to most closely follow BORIS RNA levels. Namely, the quiescent tissues made high yet variable levels of both transcripts. However, BORIS rises from proliferating to involuted hemangiomas (p = .05) while IGF2 drops precipitously. This suggests that another factor, aside from BORIS, may be regulating IGF2 gene expression. One explanation of the paradoxical fall in IGF2 levels, despite increasing levels of a factor that is associated with loss of imprinting of IGF2 [67], is the highly significant increase in CTCF (Figure 1A). From proliferating to involuting lesions, the CTCF transcript is upregulated approximately 13 fold (p = .0009.) Furthermore, older lesions also produced significantly more CTCF protein as well (Figure 1D.) In fact, the transcript difference between these two rival factors is most predictive of IGF2 RNA levels: the CTCF to BORIS transcript difference mirrors IGF2 expression over all age ranges (Figure 2A) as higher relative levels of BORIS coincide with increased IGF2. As lesions age, CTCF increases relative to BORIS and IGF2 levels decline (Figure 2A.) By directly correlating the CTCF-BORIS difference and IGF2, the relationship is made explicit (Figure 2B.) With very high correlation (R^2 = .976), IGF2 RNA is demonstrated to be inversely related to CTCF and positively correlated to BORIS. Yet, on this graph there are two distinct CTCF-BORIS vs. IGF2 curves (Figure 2B.) The first group of samples, marked by red triangles appears to demonstrate a far greater collective sensitivity to the relative levels of

CTCF and BORIS, with a slope that is approximately eight fold steeper than its less responsive counterpart marked by the blue squares ($Y = -1.7x + 2.3288$, $R^2 = .976$ versus $Y = -.2172x + 2.0493$, $R^2 = .976$.) This change in sensitivity to the CTCF-BORIS difference correlates with a genetic polymorphism identified both in tissue and matched patient control blood (Figure 2C, 3A,B,C,D.) A common C/T allele within CTCF BS6 corresponds with two strikingly different methylation patterns, as well at the two distinct CTCF-BORIS vs. IGF2 curves. Those lesions possessing the T allele, could not be methylated at position number 5 in CTCF BS6 because thymidine can not accept methyl carbons. Concurrently, these were the samples that displayed increased sensitivity to the relative quantities of CTCF and BORIS RNA. On the other hand, those samples with the C allele, could be methylated at position number 5 and were all part of the less responsive category. This polymorphism has previously been catalogued, rs10732516, and the two distinct methylation patterns resulting from carrying one of these alleles has been documented [33]. However, this is the first report suggesting a possible functional consequence of this epi-genetic polymorphism. Yet, as the quantitative methylation data demonstrate, the epigenetic variability of CTCFBS6 extends beyond a single polymorphism.

Multiple epi-genetic phenotypes at CTCF BS6

Hemangioma tissue, parallel to the patient matched control blood, displays six different epigenetic phenotypes (Figure 3 A,B,C,D.) As previously stated, those carrying the “C” allele at CpG position 5 on the assay (CpG site four of the Core CTCF Binding Site 6) are methylated, while those carrying the T allele at this site can not be methylated, thus the precipitous drop in methylation is explained by a known polymorphism, rs10732516.

Furthermore, regardless of CpG #5 status, the samples--be they tissue or patient matched control blood--can be grouped into three distinct methylation categories. Using the four CpG's flanking the polymorphic site in the control blood, methylation levels of each category were estimated to be: Low- 35% SD+/-= 5.13, Medium 48% SD+/-= 6.5, and High 58= SD+/- 7.74. The methylation of the hemangioma tissue did not significantly differ from the patient matched control blood: Low- 34% SD+/-= 3.11, Medium- 42% SD+/-= 3.5, High- 53.8 SD+/-= 8. These six distinct epi-genotypes are in agreement with Tost *et al*[33], who first demonstrated the phenomena in normal placental, fetal and maternal lymphocyte samples. Furthermore, Tost *et al* demonstrated that these categories reflect real biological differences rather than artificial divisions using the Monte-Carlo based permutation test. However, a functional significance, if any, of these polymorphic methylation levels of CTCF BS6 are yet to be demonstrated. Unfortunately, only nine samples were characterized with both transcriptional and matched methylation data. Of these nine, two are highly methylated, two are of the intermediate phenotype, and five are in the lowest category. Hence, the transcriptional consequences and or causes of CTCF BS6 methylation could only be investigated in the lowest methylated group.

CTCF BS6 methylation strongly correlates with the CTCF-BORIS difference, and is consistent with being a methylation sensitive activator of IGF2 in IH.

Plotting the CTCF - BORIS difference against CTCF BS6 methylation (Figure 4A) reveals that BORIS expression favors hypermethylation while CTCF favors normal imprints ($Y = -1.4123x + 31.492\%$, $R^2 = .8823$, $n = 5$.) In fact, higher relative levels of BORIS coincide with up to a 50% increase in methylation compared to control blood

samples, while in those tissues where CTCF predominates, methylation levels are within the 95% confidence interval of the control blood (34% +/- 2.5%.) Assuming that CTCF BS6 is indeed a methylation sensitive activator of IGF2 production, these data may partially explain a mechanism by which increased BORIS levels coincide with IGF2 upregulation. Namely, as previously demonstrated [66], BORIS hypermethylates CTCFBS6 which may lead to a change in chromatin conformation facilitating IGF2 expression. This hypothesis is partially confirmed by looking at the relationship between CTCF BS6 methylation and IGF2 expression directly (Figure 4B.) Here increased methylation at this site weakly to moderately correlates with increased IGF2 expression ($Y = 0.1593x - 3.1566$, $R^2 = .5718$.) The methylation status two other imprinted regions in the IGF2 locus are now presented.

DMR0 is hypermethylated in IH.

Methylation levels at DMRO--corresponding to base pair numbers 956,653 to 956,893 of the genomic contig NT_009237.17--of 21 hemangiomas, 11 matched control bloods and the average of 5 endothelial cell lines were compared (Figure 5A and B.) Cui *et al*[39] first established that DMR0 is maternally methylated and is necessary for the maintenance in IGF2 regulation. Unlike CTCFBS6, where hypermethylation leads to upregulation of IGF2, it is hypomethylation of DMR0 that coincides with increased IGF2 expression. Curiously, DMR0 is on average 14%, and in the most extreme case 20%, more methylated in IH (n = 21) than both patient matched control blood(n = 11) and the EC control lines (n = 5) and CpG #3; $P = 1x10^{-12}$. (Tissue = 66.7% SD+/- 4.3, Blood = 52.8% SD+/- 3.6, EC = 52.0% SD+/- 1.98.) Moreover, these findings were confirmed independently with semi quantitative standard sequencing methods, using different

primers to amplify the same bisulfite treated region (Figure 23 B.) From the fully quantitative pyrosequencing results, it was found that CTCF expression alone, rather than the CTCF to BORIS difference, correlated best with DMR0 methylation (Figure 6A.) Increasing CTCF expression was coincident with hypermethylation of the region: $Y = 4.2213\ln(x) + 62.968$, $R^2 = .6973$. This is consistent with Kurucuti *at el*[17] who first demonstrated that CTCF makes physical contact with this region to repress IGF2 expression. Furthermore, through a CTCF consensus binding site search engine (http://www.essex.ac.uk/bs/molonc/spa.htm) a potential CTCF binding site 25 base pairs centromeric to DMR0 has been identified (Figure 6C.) As CTCF is upregulated in IH compared to both the matched patient control blood samples and the EC lines, it is not surprising that DMR0 is hypermethylated in IH. However, the functional significance of this epi-genetic difference is not clear: the scatter plot of IGF2 versus DMR0 methylation reveals no direct relationship (Figure 6B.)

Exon 9 is focally demethylated in IH but sill serves as a potential methylation sensitive activator of IGF2.

Methylation levels at DMRO--corresponding to base pair numbers 941,505 to 941,685 of the genomic contig NT_009237.17--of 21 hemangiomas, 11 matched control bloods and the average of 5 endothelial cell lines were compared. The most striking difference between the tissue and control blood is at CpG #5 (Figure 7A). The tissue is hypomethylated compared to its matched controls (Tissue= 39% SD+/- 7.396; Blood= 56.7% SD+/-5.84; P= .00001) Furthermore, these results were confirmed independently with unique bisulfite specific primers and conventional sequencing (Figure 23 A) As exon 9 displays tissue specific methylation patterns, this difference was compared to the

average of four normal endothelial cell control lines to further confirm that these results are hemangioma specific rather than simply endothelial cell specific. The only remaining CpG that was statistically significant when comparing hemangiomas to both control blood and control cell lines was CpG #5 (P Tissue vs. Blood = .00001; P tissue vs. EC = .005.) Although the endothelial cell lines appear to be hypermethylated compared to control blood, this difference did not reach statistical significance (CpG #5: average of Cell lines 77% SD+/- 6.04, average of blood 56.7% SD+/-5.84; P = .09.) In addition the focal demethylation of exon 9 was confirmed qualitatively using different bisulfite specific primers and conventional sequencing as well. It is perhaps not a coincidence that CpG#5 is 10 base pairs centromeric to a potential CTCF binding site--identified by the search engine listed above--as methylation at CpG#5 strongly correlates with the CTCF-BORIS difference (Figure 7C.) However, two distinct methylation versus CTCF-BORIS curves are evident; and most intriguingly, the samples composing each curve can be differentiated by the C/T polymorphism at CTCF BS6 (Figure 9A.) In both curves, higher relative levels of BORIS correspond with increased methylation, while increased levels of CTCF favor less methylation. Yet, the "T" allele demonstrates far greater responsiveness to the CTCF- BORIS difference. These data indicate that the genotype of CTCF BS6 strongly affects the magnitude of the potential effects of CTCF and BORIS on Exon 9 (See Fig. 3A vs 3B and 9A.) What is interesting about these results is that that CTCF BS6 is 80KB distal to Exon 9. Although a physical interaction between Exon 9 and CTCF BS6 was previously demonstrated in murine models[17] (Figure 12), the concordance in hemangioma tissue between two distinct Exon 9 methylation curves and a polymorphism 80 KB distal in CTCF BS6 is to date the most direct evidence that these two regions interact in human tissue. Furthermore, this interaction likely plays a

significant role in regulating IGF2. When plotting Exon 9 methylation against IGF2 production, two very similar curves emerge (Figure 9B.) However, in this case the "C" allele is the most dynamically regulated: an apparently subtle change in methylation from 34% to 39% correlated with an 11 fold increase in IGF2. The "T" allele demonstrated a similar increase in IGF2 but over of a much broader range of 25% to 52% methylation. It is remarkable that both alleles, C or T, operate over different but complementary dynamic ranges. For the T allele, exon 9 methylation significantly changes in response to CTCF and BORIS, but IGF2 production is far less responsive to these changes. Conversely, the C allele operates under a far narrower dynamic range of exon 9 methylation, but IGF2 production changes sharply given these relatively subtle alterations.

Evidence of cooperativity between CTCF BS6, Exon9 and DMR0 in regulating IGF2

Note: Due to limited sample size, the following data is limited to samples that exhibited the "Low" methylation phenotype at CTCF BS6. Current imprinting models implicate DMR0 as a methylation sensitive inhibitor of IGF2, while Exon 9 and CTCF BS6 are methylation activating (Figure 11.) When examined independently in IH tissue, DMR0 bore no correlation to IGF2 expression (Figure 10A.) Furthermore, Exon 9 and the ICR only correlated moderately well, but with p values that did not reach significance: R^2 = .7076, p = .0741 and R^2 = .5718 p = .139177 respectively (Figures 10B and 10C.) Assuming that exon 9 activates IGF2 via increased methylation and DMR0 represses expression through the same process, taking the ratio (Exon9/DMR0) between the two methylation values greatly improves the correlation with a significant P value: R^2 = .8794, p = .018 (Figure 13.) Although DMR0 did not regulate IGF2 independently, these data suggest that in conjunction with Exon 9, it is indeed a methylation sensitive

repressor of IGF2 in IH. Similarly, assuming that both Exon9 and the ICR are methylation sensitive activators of IGF2, taking their product results in similar improvements in the correlation and p value: R^2 = .875, p = .019. Yet, the strongest correlation and most significant p values were obtained by taking all three regions into account by calculating the methylation product of exon9 and CTCF BS6 divided by DMR0: R^2 = .9005, p = .013 (Figure 13.) This data supports the hypothesis that all three imprinted regions within the IGF2 gene cooperatively regulate its expression, not only in mouse, but also human tissue.

Progressive Demethylation of the H19 Promoter Correlates to Transcript Upregulation in IH.

Two CpG's within the H19 promoter region immediately upstream to the transcription start site were analyzed for methylation status--base pair numbers 806,386 and 806,397--genbank gi:51470970--Figure 14A and 14B) Hemangioma samples (21) were significantly hypomethylated compared to 11 matched control blood samples as well as 4 endothelial cell lines (Hemangioma: 27.1% SD+/- 5.26, Blood: 57.6% SD+/- 3.98. P = .0002; EC 52% SD+/- 7.2 $P_{\text{hemangioma vs. EC}}$ = .03.) Furthermore, as the lesions age, regression analysis suggest that the H19 DMR progressively demethylates over time (Y = -3.4852lx(x) + 48.474, R^2 = .7782.) These findings were confirmed with a genomic Southern analysis of the same CpG site (Figure 21 and 22): Hemangioma 25.5% SD+/- 4.2, Tissue 54% SD+/- 3, P = .00001; EC 49% SD+/- 5 (p tissue vs EC = .042;) with the progressive demethylation curve Y = -5.513lnx +52.92 R^2 = .8257.

It is interesting to note that the same putative CTCF binding sequence "GGGAGGGG" found near DMR0 straddles the H19 transcription start site (Figure 14C.) In addition, as CTCF alone correlates best with hypermethylation of DMR0 (Figure 6A), CTCF expression correlates most strongly with the demethylation of the H19 promoter (Figure 15A) ($Y = -5.6484\ln(x) + 27.295$, $R^2 = .914$). These data suggests that in IH, the GGGAGGGG site near both DMR0 and the H19 promoter may be under the sole influence of CTCF. In turn, promoter methylation correlates inversely with H19 expression ($Y = -3.4206\ln(x) + 13.396$, $R2 = .7935$) (Figure 15B.) One potential explanation for these data is that CTCF upregulates H19 expression not only by binding to CTCF BS6 but also by contributing to the demethylation of its promoter. As H19 is a putative tumor suppressor, this would be congruent with the anti-tumorigenic function of CTCF.

CTCF and BORIS may be regulated by similar Micro RNAs in IH

To investigate the potential role of micro RNAs in the pathogenesis of IH, a pilot micro RNA array study was undertaken. All human micro RNA sequences currently catalogued at the Sanger 4.0 database were probed. A list of statistically significant micro RNAs were compiled by investigating the micro RNA transcriptome from five hemangiomas across an age span of 95, 420, 547, 760 and 1520 days against two normal endothelial cell control lines, HUVEC and HDMEC. Any one micro RNA that varied with a p value of less than .01 from one sample to any other was considered significant (Figure 16.) This reduced the number of potentially important Micro RNAs from 470 to 167. Secondly, a search for micro RNAs that target CTCF or BORIS was performed by

using the three major target prediction programs: PicTar, TargetScan, and Mirbase (http://www.pictar.bio.nyu.edu; http://www.targetscan.org; http://microrna.sanger.ac.uk/sequences). As micro RNA target prediction is an emerging computational science, only those RNAs that were predicted to target CTCF or BORIS by at least two independent algorithms were examined (Note: BORIS target predictions were only available from MirBase and TargetScan.) This further reduced the number of candidate micro RNAs from 167 to 15. Lastly, only those micro RNA's from this group that were statistically significant were considered regulatory candidates of CTCF and BORIS (Figure 17 .) By regression, these six micro RNA's were correlated to the CTCF or BORIS transcript level that they putatively target. Intriguingly, two related Micro RNAs on different chromosomes—mir-23a on chromosome 19 and mir-23b on chromosome 9—targeted both CTCF and BORIS by the majority of prediction algorithms (Figure 17.) Furthermore, these two highly similar micro RNAs correlated strongly with the downregulation of both CTCF and BORIS (Figure 18 and 19.) Nonetheless, despite the structural similarities between these two RNAs, there was one significant A/U variation at position 18 near the 5' phosphate with potential functional consequences (Figure 19.) The effects of this variation were negligible regarding CTCF expression (Figure 18), as each correlated with the downregulation of CTCF equally well (Mir-23a: $Y = -.0003x + 2.533$, $R^2 = .6317$; Mir-23b: $Y = -.0003x + 2.525$, $R^2 = .5546$.) However, as the U in 23b formed an extra bond with CTCF compared to 23a (Figure 19), 23b strongly correlated with the downregulation of CTCF while BORIS appeared unaffected: (Mir-23a vs. BORIS $Y = -12.02\ln(x) + 106.3$, $R^2 = .7047$; Mir-23b vs. BORIS $Y = -10.282\ln(x) + 90.693$, $R^2 = .3385$.) When comparing the data first by micro RNA rather than CTCF or BORIS, another potential pattern emerges. Mir23a appears to

correlate with BORIS downregulation more strongly than CTCF (R^2 = .6317 versus R^2 = .7047,) while mir23b appears to correlate with CTCF downregulation more strongly than BORIS (R^2 = .5546 versus R^2 = .3385.) From this data, it appears that both of these micro RNAs may downregulate CTCF and BORIS, yet the extent by which they do so could vary. It may be that mir23a expression favors higher levels of CTCF relative to BORIS while mir23b expression favors the opposite. Thus, the relative amounts of 23a and 23b may contribute to determining the relative levels of CTCF and BORIS. This hypothesis was further tested by plotting the difference between mir-23a and 23b against the difference of CTCF and BORIS (Figure 20.) These results strongly indicate that increases in mir-23a favor CTCF, while increases in 23b favor BORIS: Y = .0067x - 5.5377, R^2 = .9299 (Figure XB.) This is the first data suggesting that not only are micro RNAs imprinted[88], but micro RNAs may also affect imprinting itself by moderating the relative levels of chromatin organizing proteins.

Summary of Results:

- **Identified a Strongly Oncogenic Transcript BORIS in IH**
  - Likely proliferative factor
  - First non-malignant neoplasm identified expressing ectopic BORIS

- **13 Fold Increase in CTCF from Proliferating to Involuting IH**
  - CTCF is a growth suppressive factor and BORIS antagonist
  - Likely involuting factor

- **Confirmed the Presence of BORIS and CTCF at the Protein Level**
  - Significant increase in CTCF from youngest to older samples
  - BORIS is expressed throughout the age range but appears higher in younger samples

- **CTCF – BORIS is highly predictive of IGF2 Transcript levels**
  - First to quantitate CTCF and BORIS precisely enough to correlate with IGF2
  - Likely mechanism is altered DNA methylation of IGF2/H19 imprinted regions

- **First Identified Genetic Abnormalities Associated with Non-Familial IH**
  - Progressive Hypomethylation of the H19 DMR
    - Strongly associated with CTCF transcript levels alone
    - Progressive:
  - Hypermethylation of DMR0
    - Strongly associated with CTCF transctipt levels alone

- **First Data Suggesting that CTCF-BS 6, Exon 9 and DMR0 Cooperate to Regulate IGF2 in Humans (Previously Demonstrated in the Mouse)**

- **Identified Potential Functional Significance of a Common C/T polymorphism in CTCF BS 6**
  - T allele more sensitive to chromatin modifying effects of CTCF – BORIS
  - C allele more resistant
  - May explain clinical variability of the lesion
  - Polymorphism also identifiable in patient blood allowing for easy clinical testing

- **Identified a Pair of Micro-RNAs Targeting Both CTCF and BORIS**
  - Mir23a favors CTCF while Mir23b favors BORIS
  - First data suggesting that Micro RNAs affect imprinting
    - Suggests a possible clinical therapy for BORIS positive tumors via exogenous dosing of anti-MIR23b siRNA or MIR23a facsimiles

DISCUSSION

Hemangiomas are unique within the spectrum of human tumorigenesis. Unlike most highly proliferative lesions, hemangiomas eventually regress in lieu of malignant transformation[2, 3, 7]. The benign nature of IH is particularly surprising in light of the fact that hemangiomas are BORIS positive. Until now, BORIS expression outside the testes is invariably associated with malignancy--80% of lymphomas, breast, osteosarcoma and melanoma, among others [47, 59, 92]. This places hemangiomas in yet another highly unusual category--the first benign tumor that is BORIS positive. In view of these facts, perhaps the most germane question to this discussion is not how hemangiomas begin, but rather why they end.

Given that CTCF is a proven tumor suppressor [57], the thirteen-fold increase observed from proliferative to involuting lesions, suggests one potential explanation. To date, a change in transcript level of this magnitude is the most significant yet identified in IH. Furthermore, CTCF's pivotal role in maintaining the imprinted regulation of IGF2 and H19 is supported by a decade of research [16, 17, 19, 24, 29, 31, 40, 45, 47, 56, 67]. Elevations in CTCF may explain the reciprocal decline of IGF2 (mitogenic factor) and rise in H19 (tumor suppressor.) Although the exact role of H19 is yet to be defined, the fact that mir-675 stems from H19 suggests new roles for this functional RNA [78]. Levels of H19 increase two fold in IH while its promoter is progressively demethylated; the role of H19 in the pathogenesis of IH could be germane to the topic of involution. A quantitative

RT PCR specifically for mir-675 is currently underway. This is in conjunction with mir-675 transfection studies in hemangioma enriched EC cultures documenting any potential effects of this newly discovered micro RNA.

Thus far, CTCF and BORIS have been addressed individually. However, it is important to note that in IH they are co-expressed and both at abnormally high levels compared to EC controls. To further complicate matters, experimental evidence suggests that CTCF and BORIS oppose one another functionally: the IGF2/H19 is a classic example of their potential rivalry. As CTCF is binding is necessary to stabilize methylation levels at CTCF BS6 [19, 35, 38], ectopic BORIS likely methylates those same regions [66]. Taken one step further, CTCF and BORIS are likely competitive regulators IGF2. In abnormal tissues expressing both transcripts, IGF2 transcript levels should reflect a function of both CTCF and BORIS. The transcriptional results support this notion in IH. Although the ratio between CTCF and BORIS was predictive of IGF2 production (data not shown), it was the difference between these two rival siblings that correlated best with IGF2. Moreover, the CTCF – BORIS difference also revealed another interesting phenomenon. A polymorphic site within the critical CTCF BS6 separated the samples into two distinct groups. The “T” allele conferred a far greater sensitivity of IGF2 production to relative levels of CTCF and BORIS. On the other hand, the “C” allele was far less responsive to alterations in the CTCF – BORIS difference. As the relative difference between these two transcripts is dynamic in IH, this polymorphism may be of clinical importance. The implication being, that the “T” and “C” alleles may consistently produce different levels of IGF2 in IH. Moreover, IGF2 overproduction is a major factor determining resistance to apoptosis and aggressiveness in multiple tumor models and clinical settings [93-95].

Specifically to IH, the potential allelic difference may impact such clinical variables as tumor size, aggressiveness, or time to involution. A prospective clinical investigation to explore the potential genotypic effects of the C/T polymorphism is nearing completion. Furthermore, the results of which could potentially be extrapolated beyond IH to other BORIS positive tumors with deranged IGF2 axes: melanoma, breast, osteosarcoma, and 80% of leukemia [67, 96, 97]. Most conveniently, regardless of tumor type, testing a person's carrier status could be performed with a simple blood test.

It is important to note that all hemamngioma samples—be they proliferative or otherwise—demonstrate epi-genetic characteristics of IGF2 repression. DMR0 (a methylation sensitive repressor) was hypermethylated above the expected 50% as the matched control blood and endothelial cell control lines were normal. Moreover, Exon 9 (a methylation sensitive activator) focally demethylated below the expected 50% level of methylation; in addition, both the EC controls and matched blood controls were methylated slightly above the expected values. Taken alone, these epi-genetic alterations would suggest repression of IGF2. Yet, clearly this is not the case. From these facts, two questions arise: 1) What factors are responsible for the changes in methylation and 2) How can IGF2 levels in IH be so dramatically elevated despite the predominance of this repressive methylation.

1) Factors that may be responsible for repressive methylation at IGF2: A potential answer stems from appreciating that both BORIS and CTCF are upregulated in IH. Not only did CTCF increase 13 fold from proliferative to involuted lesions, but it also began at a level 5

fold higher than EC control lines. The potential effects of increased CTCF on DNA methylation is suggested by correlating the two. Hypermethylation at DMR0 correlated strongly with the level of CTCF transcript alone; complementary to this data, only CTCF levels, independent of BORIS, correlated with demethylation of the H19 promoter. Perhaps it is not coincidence that these two imprinted regions are adjacent to the identical "GGGAGGGG" sequences that can bind CTCF. Though the 11 zinc fingers shared between CTCF and BORIS are identical, the intercalating amino acid sequences are not [59]. These differences may confer subtly dissimilar binding spectrums for CTCF and BORIS. Possibly, GGGAGGG sites favor CTCF binding over BORIS. This hypothesis is being tested using chromatin immuno-precipitation techniques that will 1) confirm, or refute, that these native sequences bind CTCF or BORIS and 2) quantitate the relative abundance of CTCF and or BORIS binding. As for the focal demethylation at Exon 9, a preponderance of CTCF in early samples may be responsible. A parallel example of the H19 promoter may demonstrate this point. In the samples tested, the H19 promoter demethylates steadily over the course of time. Although the earliest time point available for study is three months, the projection of this curve back to time point zero, indicates that children are born with normally methylated DNA. If ultimately true, this fact would suggest that abnormal methylation patterns in IH are acquired after birth. Furthermore, as CTCF was the only factor found to correlate with demethylation of the H19 promoter, these findings may be generalizable to other regions of H19/IGF2. Currently, samples from younger patients to confirm this projection at H19 are being sought. Moreover, these younger samples may potentially reveal progressive chromatin changes at Exon 9 and DMR0 that may take place more proximate to birth.

2)IGF2 upregulation in IH despite the predominance of repressive methylation: Kurukuti *et al [17]* demonstrated in murine models, as the IH data supports in the human, that IGF2 activation and repression are not functions of any single imprinted region. Rather, Exon 9, DMR0 and CTCF BS6 form a cooperative network of gene regulation. In the tissue tested, although methylation at Exon 9 and DMR0 would indicate repression of IGF2, CTCF BS6 (perhaps the most important methylation sensitive activator) was not hypomethylated compared to matched patient blood controls. In fact, samples making a predominance of BORIS were as much as 50% more methylated than their CTCF rich counterparts. Exon 9 responded in kind to elevated levels of BORIS with up to a 100% increases in methylation compared to those samples transcribing more CTCF. In this way, BORIS may potentially upregulate IGF2 by working around areas of focal repression, taking advantage of the cooperativity between locally imprinted regions. Furthermore, recent evidence indicates that BORIS also works as a transcription factor by recruiting Sp1 to promoter sites, a capability that CTCF lacks [98]. Interestingly, software analysis (matinspector v2.2, www.geomatix.de) indicates numerous Sp1 binding sites within Exon 9 and DMR0 of IGF2. Thus, it is possible that heightened levels of IGF2 in IH, are caused by the expression of BORIS, working either through chromatin remodeling, transcription factor recruiting, or both. Currently, this line of research is being investigated by in vitro transfection experiments of hemangioma derived EC cultures with CTCF and BORIS. Furthermore, chromatin immuno-precipitation analysis will confirm the level of respective CTCF and BORIS binding at the sites of interest. However when plotting IGF2 expression against the difference between CTCF and BORIS, this study offers persuasive correlational evidence suggesting that more than 97% (R^2 = .976,) of the variation in IGF2 can be explained by the difference in CTCF to BORIS. This makes

BORIS a potentially attractive therapeutic target in IH. Loukinov *et al [92]*, demonstrated prolonged survival in a BORIS positive murine tumor model following administration of a "BORIS tumor vaccine." Such BORIS vaccines may be feasible in female patients suffering from IH, as BORIS to date is not transcribed in normal female tissue [59]. However, an anti BORIS vaccine may disrupt fertility in males. Yet, the testes are an immune privileged site and may be unaffected by such therapies.

As the potential consequences of CTCF and BORIS co-expression in IH have been investigated, the question remains: What may be causing the inappropriate expression of CTCF and BORIS to begin with? Normal transcription of BORIS is strictly limited both spatially and temporally to primary spermatocytes, soon to become silenced upon reactivation of *CTCF* in postmeiotic germ-line cells [59]. The mutually exclusive expression profile of CTCF and BORIS in normal tissue begs the question: how are these two respective chromatin organizing proteins reciprocally regulated in the first place? Several potential regulators of CTCF have been proposed including poly-ADP-ribosylation and phosphorylation [51, 99-101]. However, no candidate genes regulating BORIS are yet identified. A novel approach to this question was to, for the first time, investigate the micro RNA transcriptome of IH. This analysis was further informed by consulting the three primary micro RNA target prediction programs available to the public. Quite amazingly, one set of related micro RNAs, mir-23a and mir-23b, was predicted to target both CTCF and BORIS. Furthermore, these two micro RNAs were differentially regulated from proliferative to involuting hemangiomas. The preliminary data comparing two EC control cell lines against five hemangioma samples indicates that both mir-23a and 23b are downregulated by 66% compared to normal ECs.

Concordantly, both CTCF and BORIS were strongly upregulated in matched samples. If further testing confirms this finding, then downregulation of micro RNA 23a and 23b collectively could be central to the upregulation of CTCF and BORIS, which may figure largely in pathogenesis of IH. However, collective downregulation does not address the relative levels of CTCF and BORIS in a given tissue sample. Upon closer inspection, it was found that mir-23a preferentially inhibited BORIS while mir-23b preferentially inhibited CTCF in IH. Furthermore, the difference between mir-23a and 23b correlated strongly with the difference between CTCF and BORIS: Higher relative levels of mir-23a favored CTCF while higher relative levels of 23b favored BORIS. By regression, the difference between 23a and 23b, with an $R^2 = .9299$, explained over 90% of the variation in the CTCF – BORIS difference. This microarray study is expanding to include ten more samples with quantitative RT PCR for mir-23a and mir-23b to validate the results. The next step will be to confirm the effects of mir-23a and 23b in BORIS positive hemangioma cell cultures. By explaining CTCF and BORIS disregulation by a mechanism of micro RNA, a number of small molecule therapies may be available. As proof of principle, Krutzfeldt *et al [102]* stably knocked down mir-122 in mouse livers using a liver specific targeting system and chemically stabilized antisense ribonucleotides, essentially using an exogenous micro RNA against an endogenous one. They demonstrated long lasting downregulation of mir-122 with stable upregulation of most of the computed targets of mir-122. Furthermore, inhibition of mir-122 produced the expected phenotype of impaired cholesterol biosynthesis. It is likely that CTCF and BORIS are central to the pathogenesis of IH (not to mention sundry malignancies). If indeed they are regulated by mir-23a and 23b, then targeting mir-23b in endothelial cells may establish a CTCF to BORIS difference favoring involution. This would offer

unparalleled gene specificity as well as tumor specificity if such a therapy were encapsulated in target specific vectors using hemangioma selective markers such as Glut-1, CD 34 or the Lewis Y antigen [6].

Within the gamut of human tumors, hemangiomas are unique. Studying the pathogenesis of IH is an opportunity to compare dynamic molecular processes against an equally dynamic but predictable disease course. Central to these findings is that IH pathology is epi-genetically related to IGF2 and H19. However, this is one imprinted locus among many. It will be important to quantify the potential effects of CTCF and BORIS coexpression on other potentially sensitive regions. Fortuitously, the original micro-array paper implicating IGF2 as an important factor in IH, also offers a glimpse into this new potential line of research [8]. Although not specifically addressed by the authors, the supporting micro array data indicates that DLK, another imprinted gene, is downregulated four fold from proliferative to involuting lesions. Furthermore, like IGF2, DLK is paternally transcribed with growth promoting potential of its own. Moreover, as DLK is a potent inhibitor of adipogenesis, the fact that hemangiomas involute into fatty tissue as DLK is downregulated merits further investigation.

Another important challenge facing human epi-genetics is to identify the regulatory networks controlling CTCF and BORIS themselves. Here, IH offers important clues as well. CTCF and BORIS may be partially regulated by micro RNAs. Mir23a and 23b appear to correlate not only with the total level of CTCF and BORIS but also the difference between them. Recently, it has been demonstrated that certain micro RNAs

within the DLK locus, are also imprinted [88]; one of them, mir-299, is computed to target CTCF. The possibility that these micro RNAs are regulated by and are potentially regulators of CTCF and BORIS is currently being investigated. These data offer a new and yet to be verified conceptual model of imprinting and growth, implicating feedback networks between chromatin modifying proteins, imprinted genes and the micro RNA transcriptome. As such, IH offers a singular opportunity to study human epi-genetics as the disease course itself, from proliferation to involution, titrates potential epi-genetic modifiers in a predictable fashion. In this way, IH can be thought of as a natural experiment in chromatin remodeling just beginning to be explored.

Summary of Future Directions

Experiment	Status
Loss of imprinting studies using allele specific RT PCR of polymorphic exons in H19 and IGF2: This will confirm biallelic versus monoallelic expression of IGF2 and/or H19	Assays designed and DNA Genotyped: hetrozygotes identified, will perform analysis of cDNA forthwith
Loss of Function Experiments: Knockdown of CTCF and BORIS in hemangioma derived Endothelial cell cultures with siRNA--Followed by FACS analysis for hemangioma specific markers, proliferation assays, DNA methylation analysis transcriptional quantitation and protein analysis.	In progress: Collaborating with the labs of Dr. Jordan Pober and Dr. Ruth Halaban
Gain of Function Experiments: BORIS Transfection of Normal HDMEC cells--Followed by FACS analysis for hemangioma specific markers, proliferation assays, DNA methylation analysis transcriptional quantitation and protein analysis.	In progress: Collaborating with the labs of Dr. Jordan Pober and Dr. Ruth Halaban
Retrospective Study of the C/T polymorphism effect on Hemangioma growth using 21 previously epi-genotyped samples	Nearing completion

Prospective Clinical Study Evaluating the effectiveness of the C/T polymorphism as a clinical test predicting time to involution and tumor size in IH	Planned to begin April, 2007: in Cooperation with Dr. Milton Waner of the Vascular Birthmarks Foundation of New York
Expansion of all IH tissue experiments including the micro RNA array studies with 10 more samples: IGF2 ELIZA and further quantitative RT PCR for Mir-675, Mir-23a, Mir-23b and IGF1 and 2 receptors.	Sample Collection ongoing: Expected date of completion, April, 2007
Transfection of BORIS positive melanoma cell lines with Mir 23a, Mir23b and their respective antagomers followed by quantitative RT PCR for micro RNAs, CTCF, BORIS and IGF2, with FACS phenotyping	Planned to begin May, 2007: In cooperation with the lab of Dr. Ruth Halaban
Animal Trial of IGF2 signal modulation using injectable poly-L-lactide carriers impregnated with Rapamycin, an IGF2 signal transduction antagonist.	In cooperation with the Dr. Mark Saltzman Lab (Yale department of Biomedical Engineering.)

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