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Hemangiomas of Infancy: Mesenchymal Stem Cell Tumors of Perivascular Origin

Christopher Spock

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Christopher Ryan Spock

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Abstract

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Introduction: Hemangiomas of infancy (HOI) are the most common benign tumor of childhood. Initially thought to be composed entirely of endothelial cells, it has recently been shown that mesenchymal stem cells reside within these tumors. We propose that hemangiomas represent mesenchymal stem cell tumors of pericyte origin, as demonstrated by expression of pericytic markers (NG2, PDGFR-β, and DLK), neural crest origin (expression of nestin and sox10), expression of factors that play a role in the maintenance of stem cell pluripotency (Oct4, Sox2, Nanog, C-myc, and piRNAs), and a microRNA expression profile suggestive of mesenchymal stem cells.

Methods: Quantitative RT-PCR was performed on 19 hemangioma specimens (4 proliferating, 10 plateau, 5 involuting) analyzing transcription factors (Oct4, Sox2, C-myc, and Nanog) known to regulate stem cell pluripotency. Transcription factors RB, IGF2, CTCF, BORIS, DLK, and CDX-2 were also examined. PiRNA analysis was performed on 7 hemangioma specimens to investigate the role of these small RNA transcripts that interact with Piwi proteins expressed in the germline and stem cells. MicroRNA microarray analysis was performed on 9 hemangioma specimens. MicroRNA pathway analysis was performed using Ingenuity Pathway Analysis software and MetaCore Software. Freshly resected hemangioma specimens were cultured in embryonic stem cell media with and without recombinant human basic fibroblast growth factor (rhbFGF), recombinant human transforming growth factor β (rhTGF-β), and 17-β estradiol.

Results: All hemangiomas expressed factors RB, Oct-4, Sox-2, Nanog, C-myc, DLK, IGF-2, and CTCF at higher levels than endothelial cell controls. DLK, a gene that functions as a negative regulator of adipocyte differentiation, is increased in hemangiomas more than 10^5 orders of magnitude compared to control endothelial cells. MiRNA-195, which is known to target DLK, is also upregulated in hemangiomas. PiRNA analysis revealed that hemangiomas do not contain piRNA transcripts. A microRNA microarray analysis using adult and neonatal dermal endothelial cells as controls indicated that microRNAs associated with mesenchymal stem cells (miR-143, miR320a, miR320c, let-7c) were expressed in 9 samples studied. Hemangioma growth in culture was not observed in embryonic stem cell media with or without supplementation with bFGF and TGF-β. Growth was observed in standard culture media with 17-β estradiol supplementation.

Conclusion: Hemangioma specimens in all stages of growth express transcription factors and genes known to play a role in the maintenance of stem cell pluripotency at a level greater than control endothelial cells. Specifically, transcription factors Oct-4, Sox-2, Nanog, and C-myc are increased. Furthermore miR-143, miR320a, miR320c, and let-7c (all microRNAs identified in mesenchymal stem cells) are upregulated in hemangioma tissue in all stages of growth. DLK, a gene that functions as a negative regulator of adipocyte differentiation, is increased in hemangiomas compared to controls. The downregulation of this gene may lead to transformation of mesenchymal stem cells into adipose tissue. Future experiments are necessary to confirm this role.

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Statement of Purpose:

Infantile hemangiomas possess interesting and complex characteristics that are relevant to the fields of vascular biology, stem cell biology, oncology, immunology, and dermatology among others. While a great deal is known about the clinical presentation and natural history of these lesions, the exact cell of origin and the mechanisms of pathogenesis of these lesions are not well understood.

Initial theories hypothesized that hemangiomas were of placental origin. As important differences between hemangiomas and placental tissue were uncovered, the search for the cell of origin turned to endothelial cells. Increased endothelial progenitor cells are seen in these lesions, and other studies suggest that hemangiomas represent clonal expansions of endothelial cells. The composition of hemangiomas is not limited to endothelial cells; rather, mesenchymal stem cells play an important role in these lesions and may contribute to the fibro-fatty residuum left behind when these lesions involute.

The nature of the mesenchymal stem cells in hemangiomas is not well understood, and neither is the relationship between mesenchymal stem cells and endothelial cells. Recent studies have shown that mesenchymal stem cells arise from the perivasculature, specifically from pericytes, and can differentiate into a variety of tissues including adipose. The working hypothesis of this research is that hemangiomas represent aberrantly differentiated mesenchymal stem cells of a perivascular location and pericyte lineage (CD146+, NG2+, PDGFB+, and DLK) that lead to a proliferation of endothelial cells due to local hypoxia and paracrine effects of secreted growth factors.

In order to investigate this hypothesis and classify the nature of the mesenchymal stem cells in hemangiomas quantitative RT-PCR was performed on hemangioma specimens analyzing various transcription factors (Oct4, Sox2, C-myc, and Nanog) known to regulate stem cell pluripotency. Additional genes and transcription factors known to play a role in hemangioma growth (IGF2, CTCF, BORIS, H19) were studied as well, to see if there was a difference between hemangiomas in various stages of growth compared to endothelial cell controls. Additional analysis of hemangioma specimens was done using a microRNA microarray. MicroRNAs are small RNAs that have emerged as potent regulators of gene expression. Analysis of microRNAs has been used to classify various cancers and to elucidate molecular pathways involved in the pathogenesis of such lesions. We performed a microRNA microarray assay to classify the expression profile of hemangiomas and to identify new pathways that could be involved in the pathogenesis and life cycle of hemangiomas.

A related set of small RNAs that play an important role in stem cell pluripotency, known as piRNAs (named because of their interaction with Piwi-proteins) were analyzed. We searched for the presence of piRNAs in hemangiomas because piRNAs are associated with maintenance of stem cell pluripotency.

Finally, an attempt was made to develop a new culture-based hemangioma model using stem cell media and specific stem cell growth factors. A better understanding of how the genetics of hemangiomas affects the clinical course of disease will help physicians predict clinical outcomes and tailor therapies to their individual patients.

Introduction:

Classification

Hemangiomas of infancy (HOI) are the most common benign tumor in children. Historically, the nomenclature of hemangiomas has been confusing, but the classification system, described by Mulliken and Glowacki and refined by the International Society for the Study of Vascular Anomalies, provides the best framework for understanding hemangiomas and other vascular anomalies in infants and children.[1,2] Within this framework, vascular anomalies are classified as either hemangiomas or vascular malformations on the basis of cellular features in relation to their clinical appearance and natural history.

It is currently believed that hemangiomas are vascular tumors composed of hyperplastic vascular endothelial cells that have the capacity to proliferate excessively. Regarded as neoplasms, they grow by rapid endothelial proliferation and are characterized by hypercellularity.[3] Hemangiomas are often absent or small at birth, but then grow rapidly in early infancy during what is known as a proliferative phase. Then, for an unknown reason, these tumors undergo regression and involution as part of their normal life cycle. As a hemangioma involutes, fibro-fatty tissue is deposited in its place,[4] but the origin of this tissue has yet to be determined.

Immunohistochemical markers are used to identify hemangiomas, and GLUT1, a glucose transporter normally expressed in the microvascular endothelium of blood-tissue barriers such as brain, retina, placenta, and endoneurium, but not normal skin, is a specific marker for hemangiomas of infancy in all phases of development. GLUT-1 is strongly expressed in the entire endothelial lining of hemangioma vessels, but it is absent

in other vascular lesions, such as malformations, pyogenic granulomas, granulation tissue, as well as rapidly involuting and non-involuting congenital hemangiomas.[5] It is therefore used to distinguish hemangiomas from other lesions.

Hemangiomas are sub-classified into hemangiomas of infancy (HOI), rapidly involuting congenital hemangiomas (RICH), and non-involuting congenital hemangiomas (NICH). Both RICH and NICH present fully-developed at birth and remain static or rapidly involute. They are histologically and immunohistochemically distinct from HOI, most notably because NICH and RICH do not test positive for GLUT-1. The experiments described in this thesis relate specifically to hemangiomas of infancy. Therefore, the term hemangioma will be used to refer only to HOI and not RICH or NICH for purposes of clarity throughout the text.

In contrast to hemangiomas, vascular malformations result from structural abnormalities of endothelial cells that are usually present at birth, are relatively mature at presentation, and do not exhibit a tendency towards hyperplasia with rapid proliferation. Vascular malformations display a normal pattern of growth, enlarge as the child grows, and do not spontaneously resolve.

Epidemiology

Hemangiomas of infancy are the most common tumor of childhood and affect between 10% and 12% of all Caucasian children.[6] For reasons not well understood hemangiomas are more common in females compared with males at rates of 3:1 to 5:1.[7] Hemangiomas also show a predilection for the head and neck anatomic region, and in one study of 178 hemangiomas, approximately 60% were located on the head and neck.[8]

Hemangiomas are more common in premature infants, and increased prevalence of hemangiomas correlates with both earlier gestational age and decreasing birth weight. [9, 10] In addition, chorionic villus sampling (CVS) at 9 to 12 weeks of gestation has been associated with an increased incidence of hemangiomas in infants. In one study the incidence of hemangiomas was 21% with chorionic villus sampling versus 7% with amniocentesis.[11] It should be noted, however, that a more recent study found no association with CVS.[12] The underlying reasons for these clinical characteristics: preferential location on the head and neck, increased prevalence in premature infants, and possible association with CVS, could all be related to a hypoxic insult or stimulus as the triggering factor for hemangioma development. The specifics of this hypothesis are discussed in greater detail in the section discussing the pathogenesis of these lesions.

Clinical Presentation

On initial presentation hemangiomas typically appear as an erythematous pink or red macule, a blanched spot, or a telangiectasia surrounded by a pink halo.[13] Several studies have documented that approximately 40% of hemangiomas are present at birth as a small red mark.[2,8] The cellular composition of this early macular stage is not well understood. In future studies it would be helpful to biopsy these lesions in the earliest proliferative stages to identify the cell sub-types present, and for reasons explained later, to see if genes that activate in response to hypoxia are upregulated.

The natural history of these hemangiomas of infancy is well described and involves a proliferative phase early in infancy followed by an involutional phase of variable length occurring after the first year of life.[8] Hemangiomas often reach their

maximum size by 9 to 12 months or earlier, although there is a subset that continues to proliferate for up to 18 to 24 months.[4]

Involution begins as early as a few months after lesion appearance, with the usual onset at 12 to 18 months. The process usually begins centrally in the lesion and spreads peripherally. Complete involution occurs at an estimated rate of 10% per year, and a convenient way to remember the rate of involution is to consider that 50% have involuted by 5 years of age, 70% by age 7, and 90% by age 9.[4]

During involution there is a decreased amount of endothelial cell label uptake and decreased number of mast cells, with the tissue being replaced by fibro-fatty stroma during this phase.[3] Detailed knowledge of the clinical behavior of these lesions is important because it provides clues to the underlying genetic mechanisms governing hemangioma origin and pathogenesis, two major questions that remain to be answered regarding hemangiomas. Despite our clinical knowledge one of the most important areas of research is determining the cell of origin of hemangiomas.

Hemangioma Origins – Placental Connection

Multiple lines of evidence point to similarities between placental tissue and hemangiomas. The increased incidence of hemangiomas in children whose mothers had underwent chorionic villus sampling led initial support to this hypothesis.[11] It was reasoned that the trauma of CVS increased the number of placental cells released into the circulation and thus increased the likelihood that these cells embolized to fetal vascular sites.

Further support of the hemangioma-placenta connection comes from the fact that hemangioma tissue bears striking resemblance to placental tissue. Placental vasculature and hemangioma tissue share expression of several surface markers. It has been demonstrated that both tissues express merosin, Lewis Y, FcγRII (CD32), and glucose transporter 1 (GLUT-1, also expressed on endothelium of blood-brain barrier and red blood cells).[14,15] Another group performed microarray analyses on placenta, hemangioma, and eight other normal and diseased tissues and determined that the transcriptome similarity between placenta and hemangioma exceeded that of any other tissue and mirrored that seen between normal lung parenchyma and pulmonary tumors.[16]

Interestingly, the lifecycle of the placenta resembles that of hemangiomas. As the placenta develops, angiogenesis occurs at an extraordinary rate, and it is controlled by inhibitory factors that prevent inappropriate blood vessel growth into normal maternal and fetal tissue. In order to prevent uncontrolled angiogenesis the placenta produces a soluble form of the VEGF transmembrane receptor, sFlt-1, that is found in both maternal serum and amniotic fluid and serves to inhibit angiogenesis by sequestering circulating VEGF and placental growth factor. [17,18] With the passage of the placenta, the source of sFlt-1 is removed allowing unopposed proliferation of cells responsive to angiogenic growth factors. This sequence not only mirrors the natural history of hemangiomas, but it coincides chronologically with the appearance of infantile hemangiomas in the postnatal period.

Despite these similarities between placenta and hemangioma, the embolization theory is an oversimplification of the origin of hemangiomas, and there is strong evidence contradicting it. A study by Bree et al. demonstrated that the immunohistochemical

profiles of hemangiomas and placental tissue are not exactly identical. Infantile hemangiomas failed to stain for certain trophoblastic markers such as human placental lactogen, placental alkaline phosphatase, and cytokeratins 7, 8, and 11.[19] Another study investigated maternal-fetal chimerism and demonstrated that hemangiomas are exclusively derived from the child, with no evidence of maternal origin[20] as would be expected if hemangiomas were in fact embolized placenta. The similarities between placental tissue and hemangiomas, however, have generated new ideas regarding the origins of these tumors.

Hemangioma Origins – Endothelial Progenitor Cells

Recent evidence suggests that several different types of progenitor cells may play a role in hemangioma progression. The identity of endothelial progenitor cells is controversial, however, most agree that a single marker cannot identify these cells. The likely identity of endothelial progenitor cells comes from studies[21] that have shown that cells expressing both CD34 and AC133 (CD133) can differentiate into endothelial cells *in vitro*.* Normal bone marrow and peripheral blood contain such progenitor cells that can then differentiate into endothelial cells[22, 23] through the process of vasculogenesis.

Vasculogenesis occurs through a sequence of events, typically set in motion by a hypoxic insult to peripheral tissues which then leads to recruitment of endothelial progenitor cells from the bone marrow and subsequent vascular assembly. These endothelial progenitor cells must undergo mobilization from the bone marrow stroma, followed by selective homing, extravasation, differentiation, and tubule formation,

* For a complete listing of the cellular markers discussed in this text see Image 2.

ultimately leading to new blood vessel formation.[24] Vasculogenesis is differentiated from angiogenesis in which the growth of new blood vessels occurs via extension and remodeling from existing vasculature.[25]

The endothelial progenitor cells responsible for vasculogenesis may play an important role in hemangiomas as peripheral blood collected from patients with hemangiomas demonstrated a 15-fold increase in circulating endothelial progenitor cells. Those same progenitor cells also co-expressed the known hemangioma markers GLUT1, CD32, and merosin.[26] Another group was able to demonstrate the presence of these endothelial progenitor cells in actual hemangioma specimens.[27] Eleven of twelve proliferating hemangiomas in this group's cohort demonstrated CD133+/CD34+ endothelial progenitor cells. However, no such cells were identified in involuting hemangiomas. Of note, one of the specimens in this group did not contain endothelial progenitor cells, which suggests that perhaps these cells do not play a role in all hemangiomas.

Given this data, it is unclear if increased numbers of endothelial progenitor cells are the cause or effect of hemangioma formation. One postulation is that vascular progenitors are recruited to the hypoxic environment of hemangiomas through stabilization of the global regulatory protein HIF-1α and increased expression of the downstream effectors SDF-1α and VEGF. In addition to attracting and concentrating endothelial progenitor cells within the tumor, these chemokines mediate the assembly of progenitor cells into new vascular structures.[28]

The origin of the endothelial cells in hemangiomas is an important area of investigation. Several studies have tried to determine if hemangiomas are clonal lesions.

These studies examined hemangioma clonality using X-linked human androgen receptor gene assay (HUMARA) analysis. One study analyzed cells grown in culture from seven patients, and all showed a skewing toward a single allele.[29] A subsequent study analyzed intact tissue without selection of cells and found some degree of allelic loss in 12 of 14 samples.[30] These two independent investigations suggest that hemangiomas arise as a consequence of clonal expansion from a single cell; however, it is possible that sampling constraints biased these results. It is usually only possible or practical to sample a portion of a hemangioma lesion and therefore conceivable that clonality accounts for only portions of the lesion. This argument against clonality seems even more plausible when one considers segmental hemangiomas, which are large and plaque-like. If these lesions were clonal then large numbers of cells derived from a single precursor would need to spread over a large anatomic area and expand simultaneously.

Although the clonal nature of hemangiomas is debatable it is well established that hemangiomas are not composed entirely of one cell type and while endothelial progenitor cells may play a role in these lesions, other cells should not be excluded from this discussion. Mesenchymal stem cells are another important population of cells isolated from hemangiomas, and these cells may be responsible for the unique life cycle of the tumor.

Hemangioma Origins – Mesenchymal Stem Cells

During involution the tissue at the site of the hemangioma develops into fibro-adipose tissue. Little is known about the process by which this occurs, although mesenchymal stem cells may play a role. A group recently identified mesenchymal stem

cells within hemangioma tissue that are distinct from endothelial progenitor cells.[31] This suggests that there are at least two distinct cell populations within hemangiomas.

Mesenchymal stem cells are defined by their self-renewal capability and potential for multi-lineage differentiation into adipocytes, osteoblasts, chondrocytes, myocytes, neuronal cells, and hepatocytes.[32] Mesenchymal stem cells are observed in hemangiomas more frequently than in normal skin, and their numbers are highest during the proliferative phase.[31] Furthermore, these cells exhibit a random pattern of X-chromosome inactivation indicating that they are not clonally derived.[31] The cells recovered from hemangiomas are found to have many of the characteristics of mesenchymal stem cells from other tissues, and it has been hypothesized that these cells may contribute to the generation of adipocytes during hemangioma involution.

Hemangioma derived mesenchymal stem cells expressed the cell surface markers SH2 (CD105), SH3, SH4, CD90, CD29, smooth muscle actin, and CD133 but not the hematopoietic markers CD45 and CD14 or the hematopoietic/endothelial markers CD34, CD31.[31] This is evidence to the fact that hemangioma derived mesenchymal stem cells represent a distinct population of cells from the endothelial progenitor cells (CD34+/AC133+) in hemangiomas of infancy. It should be mentioned that these cells were CD34- but flow cytometry was performed on them after they had been cultured, and recent evidence indicates that mesenchymal stem cells may lose CD34 positivity in culture.[33]

Additional evidence linking stem cells to hemangiomas was demonstrated by Boscolo et al. in an experiment that isolated stem cells from hemangioma specimens and then injected them subcutaneously into the back of immunodeficient mice. These stem

cells were able to differentiate and organize into functional vessels 7-14 days after implantation. Furthermore these vessels were CD31+/GLUT1+, and two months after implantation the number of blood vessels diminished and human adipocytes became evident.[34] Unfortunately, the specific markers used by this group to isolate stem cells is not well-described in their abstract and it is unclear what specific population of cells this group was isolating prior to implantation.

Until recently the origin of mesenchymal stem cells was not known. But, groundbreaking work has demonstrated that mesenchymal stem cells arise from the perivasculature and these precursor cells have the potential to develop into adipose, muscle, bone, and cartilage. Using lineage tracing in mice a group demonstrated that the precursor cells that give rise to adipocytes reside within the walls of the blood vessels that supply adipose tissue[35] (See Image 1). Pericytes, the smooth muscle-like cells that cover the endothelial cells of blood vessels, give rise to adipocytes. These specialized cells express the proteins peroxisome proliferator-activated receptor γ (PPAR-γ), stem cell antigen 1 (sca1), and CD34. This cell population also expresses smooth muscle actin (SMA), platelet-derived growth factor receptor-β (PDGFR-β), and neural/glial cell 2 (NG2), which are all markers of pericytes, but not perilipin, a marker of mature adipocytes. Previously the only known marker of pre-adipocytes was Pref-1 (also known as DLK-1), a cell surface protein in the epidermal growth factor family.[36] DLK-1 functions as a negative regulator of adipocyte differentiation, and we hypothesize that this protein may play a critical role in the conversion of hemangiomas into adipose tissue.

The identification of these adipocyte precursor cells has important implications regarding hemangiomas. Although Tang et al.[35] find the PPAR-γ expressing cells only in

blood vessels that supply adipose tissue, pericytes isolated from other locations have adipogenic, myogenic, osteogenic, and chondrogenic potential.[37] Most pericytes, adipocytes, myoblasts, and osteoblasts are thought to be of mesodermal origin, and in all four cases similar cell types in the head and neck appear to derive from neural crest.[38, 39] It is therefore possible that the pericyte is the cell of origin of the hemangioma. We hypothesize that hemangiomas of the head and neck are derived from neural crest pericytes (which may be uniquely sensitive to hypoxia) and that hemangiomas in other anatomic locations arise from mesoderm derived pericytes.

Hemangioma Origins – Pericytes

Pericytes closely encircle endothelial cells in capillaries and microvessels.[40] Pericytes also inhibit the division of endothelial cells via TGF-β activation.[41] If one considers the role of pericytes in hemangiomas this could be a potential explanation for why hemangiomas do not become invasive secondary to uncontrolled angiogenesis.

Pericytes can be isolated in multiple human organs including skeletal muscle, pancreas, adipose tissue, and placenta using CD146+, NG2, and PDGF-Rβ expression in the absence of hematopoietic, endothelial, and myogenic markers.[37] Neural/glial cell 2 (NG2), also known as chondroitin sulfate proteoglycan, is a proteoglycan associated with pericytes during vascular morphogenesis.[42] CD146 (aka S-endo1, Mel-CAM, Muc18, or gicerin) is an endothelial cell antigen also expressed at the surface of pericytes.[43] In addition to NG2 and CD146, all perivascular cells express PDGF-Rβ but do not express endothelial cell markers such as CD144 (VE-cadherin), von Willebrand factor (vWF), CD34, or CD31.[37] The expression of PDGF-Rβ is of particular interest because cases of

familial hemangiomas have mapped to chromosome 5q31-33 which harbors the genes for FGF receptor 4, and PDGF-Rβ.[44]

The connection between mesenchymal stem cells and perivascular cells is strengthened by the work of Crisan et al. This group demonstrated that mesenchymal stem cells are derived from perivascular cells (CD146+/NG2+/PDGF-Rβ+/CD34-/CD144-/CD31-/vWF-) on the basis that these cultured perivascular cells express all recognized markers of mesenchymal stem cells including CD10, CD13, CD44, CD73, CD90, and CD105, as well as CD108, CD109 (platelet activation factor), CD140b (PDGFR-β), CD164, CD166, CD318, CD340 (HER-2), CD349 (frizzled-9), SSEA-4, and HLA-CL.[37] Interestingly another group found that the mesenchymal stem cells isolated from adipose tissue express CD34 (but remain CD31-/CD45-) indicating that mesenchymal stem cell expression of CD34 may be rapidly down-regulated in culture.[33]

Additional studies have shown that these multipotent mesenchymal stem cells of perivascular origin serve both structural and functional roles in interactions with endothelial cells.[33] Mesenchymal stem cells and endothelial cells in co-culture exhibit preferential heterotypic assembly into vascular networks *in vitro*, which demonstrate a stability advantage in comparison with networks of endothelial cells alone. It is believed that paracrine interactions between these mesenchymal stem cells and endothelial cells, due to secreted proteins such as VEGF and bFGF, mediate this stability advantage. The role of secreted factors VEGF and bFGF is of particular importance because it has been hypothesized that the use of β-blockers in the treatment of hemangiomas of infancy works because of the downregulation of these specific factors.[45] Applying this knowledge to hemangiomas one can hypothesize that hemangiomas represent mesenchymal stem cell

tumors of perivascular origin that recruit and/or direct the assembly of endothelial cells in response to a hypoxic insult.

Mechanisms Controlling Hemangioma Growth – Role of IGF-2

In addition to determining the cell of origin of hemangiomas much work has been done to elucidate the genetic mechanisms that control the growth of these lesions. In fact, exploration into the mechanisms controlling hemangioma growth may provide further insight into the cell of origin of these fascinating lesions.

Large scale gene expression analysis of proliferating versus involuting hemangiomas has shown that insulin-like growth factor 2 (IGF-2) is highly expressed during the proliferative phase and substantially decreased during the involuting phase of hemangioma growth.[46] Ritter et al. also showed that IGF-2 promotes sprouting from intact hemangioma tissue using a human hemangioma explant model.

Human insulin-like growth factor (IGF-2) is an important mitogen which plays a role in normal fetal and postnatal growth, and also in tumorigenesis.[47] Regulation of the human IGF2 gene is very complex, both at the transcriptional and post-transcriptional level. IGF2 lies 110-200 kb centromeric to H19[48] a gene whose transcript is not translated,[49,50] but whose molecular evolution suggests a functional role for its mRNA perhaps in tumor suppression. IGF2 and H19 are reciprocally imprinted, such that H19 is transcribed exclusively from the maternal allele, and IGF2 is transcribed from the paternal allele. H19 participates in the repression of IGF2, at least in part through effects on IGF2 transcription, an effect that may contribute to its action as a tumor suppressor.[51]

A differentially methylated region (DMR) with paternally derived methylation is located 2kb upstream of H19. This region acts as an insulator when unmethylated and binding sites for CTCF (CCCTC-binding factor) are exposed. CTCF is a ubiquitous 11-zinc finger protein with highly versatile functions such as transcriptional silencing or activating depending on the context. CTCF is also known as the Regulator of Imprinted Sites (RIS) because of its ability to partition DNA into active and inactive regions by insulating genes from proximate enhancers.[52,53] CTCF possesses numerous recognized functions ranging from X chromosome inactivation to genome wide regulation, and its function is critical for normal cellular processes.[54-57] Numerous studies have shown CTCF binding to DNA to be methylation sensitive.[58-61]

Previous work by Narayan's lab investigated the IGF-2/H19 locus, specifically looking at methylation changes in the promoter region. Analysis of methylation changes at this locus and sequencing has identified a C to T polymorphism[62, 63] in this promoter region where CTCF binds. Narayan's group was the first to observe that hemangiomas with this polymorphism had a faster time to regression.[64] This is the first consistent genetic phenomenon associated with non-familial infantile hemangiomas.

Furthermore, studies in Narayan's lab identified a pathologic protein, BORIS (Brother of the Regulator of Imprinted Sites), which is normally only expressed in the testis.[65] While CTCF over-expression blocks cell proliferation, expression of BORIS in normally BORIS-negative cells promotes cell growth that can lead to transformation.[65]

As previously mentioned, IGF-2 is epigenetically regulated so that only the paternal allele is expressed in most tissues prenatally and in some tissues during postnatal life, a phenomenon referred to as genomic imprinting. Preferential loss of maternal alleles

(loss of heterozygosity) and relaxation of parental imprinting (loss of imprinting) of IGF2 occurs frequently in certain pediatric tumors such as Wilms' tumors, adrenocortical carcinomas, hepatoblastomas and rhabdomyosarcomas, and in malignant adulthood tumors (including uterine, lung, and testicular tumors) suggesting that abnormal expression of IGF2 and/or of other 11p15.5 imprinted genes has a role in the pathogenesis of these diseases. However, a study by Yu et al. showed that loss of imprinting of IGF2 is not involved in the increased expression of IGF2 in infantile hemangioma.[66]

Given that a normal pattern of IGF-2 imprinting is found in hemangiomas,[66] it is possible that an abnormal response to hypoxia could explain the high levels of IGF-2 in these lesions. Hypoxia, or low levels of oxygen, is sensed by cells, which then respond by modifying gene expression. Hypoxia-inducible factor 1α (HIF-1α) is a major transcription factor that serves as a major regulator of the cellular response to hypoxia. HIF-1α is involved in several cellular processes including regulation of differentiation and myeloid cell function,[67] regulation of genes involved in angiogenesis, erythropoiesis, energy metabolism, inflammation, and cell survival.[68] GLUT-1 and IGF-2 expression are both under control of HIF-1α and are induced by hypoxia.[69, 70]

Hypoxia is a potent stimulus for neovascularization and its effects are largely mediated by the regulatory transcription factor hypoxia inducible factor-1α (HIF-1α). Increased HIF-1α activity in turn promotes expression of downstream hypoxia responsive genes such as vascular endothelial growth factor (VEGF) and stromal derived factor-1α (SCF-1α).[71,72] Both VEGF and SDF-1α are critical for the recruitment, trafficking, and incorporation of endothelial progenitor cells into new blood vessels.[72]

It has been demonstrated that proliferating hemangiomas exhibit significant hypoxia, leading to HIF-1α upregulation/stabilization and subsequent expression of downstream targets.[73] It was also shown that in human tissues there was increased HIF-1α expression in proliferating hemangioma samples with corresponding increases in VEGF and SDF-1α. Involuting samples did not demonstrate any upregulation of HIF-1α.

The idea of a hypoxic insult leading to new vessel growth is an intriguing hypothesis because it has been shown that the location of hemangiomas is not random [74] and may be related to areas of hypoxia. This study found that facial hemangiomas occurred in regions close in proximity to lines of fusion between mesenchymal growth centers or between mesenchymal growth centers and facial ectoderm. Interestingly, the mesenchyma of the head is derived from neural crest cells.

Neural crest-derived mesenchyma might possess unique properties that make it a particularly receptive field for externally derived vascular precursors. For example, it has been shown in chick-quail chimeras that this neurally-derived mesenchyma, unlike mesoderm-derived mesenchyma, has no endogenous angioblasts and must rely on migrating angioblasts and vascular sprouts for vascularization.[75] Given the increased need for in-migration of angioblasts one can hypothesize that regions of the head may be subjected to areas of hypoxia due to lack of blood supply, which could then lead to upregulation of HIF-1α.

Mechanisms Controlling Hemangioma Growth - microRNA Regulation

The underlying mechanisms that control expression of the IGF-2/H19 locus are still being uncovered. One potential mechanism of regulation of this locus could involve

small non-coding RNAs, specifically microRNAs, which have emerged as potent regulators of gene expression at both the transcriptional and post-transcriptional levels.

MicroRNAs (miRNAs), a non-coding RNA family, are 19 to 25 nucleotide (nt) transcripts that are cleaved from 70 to 100-nt hairpin-shaped precursors. The sequences of many miRNAs are conserved between distantly related organisms, suggesting that these molecules participate in essential processes. Although the precise biological functions of miRNAs are not yet fully understood, they have diverse expression patterns and may regulate various developmental and physiological processes. Moreover, dysregulation of miRNA expression might contribute to human disease.

The first identified miRNAs, the products of the *C. elegans* genes *lin-4* and *let-7*, have important roles in controlling developmental timing and probably act by regulating mRNA translation. When *lin-4* or *let-7* is inactivated, specific epithelial cells undergo additional cell divisions instead of their normal differentiation. Because abnormal cell proliferation is a hallmark of human cancers, it seems possible that miRNA expression patterns might denote the malignant state. Indeed, altered expression of a few miRNAs has been found in some tumor types.[76] Several papers have recently been published that explore analysis of miRNAs as a way of classifying tumors.[76-78] For example, Murakami et al.[78] looked at miRNA expression to classify the degree of differentiation of hepatocellular carcinoma.

Previous work by Narayan's group identified miRNAs that would interact with transcription factors CTCF and BORIS. This work began as a search to identify an additional epigenetic control mechanism at the IGF-2/H19 gene locus. Through multiple prediction algorithms, a set of two related miRNAs (mir23a and mir23b) that potentially

target both CTCF and BORIS were identified. The implication was that miRNA could potentially affect imprinting. By correlating mir-23a and mir-23b to CTCF and BORIS levels in the hemangioma samples tested, mir-23a correlated with CTCF and BORIS downregulation equally well, while mir-23b correlated with CTCF downregulation. In addition, the difference between mir-23a and mir-23b strongly correlated with the difference between CTCF and BORIS. Data indicated that mir-23b could be a potential anti-target of BORIS relative to CTCF; thus, increased mir-23b compared to mir-23a expression would effectively lead to increased levels of BORIS over CTCF.

MicroRNAs, however, serve a diverse set of functions and analysis of miRNA expression profiles of hemangiomas could give important insight into the molecular pathways involved in the pathogenesis of these lesions. Furthermore, analysis of miRNA expression could also give insight into the cell of origin of hemangiomas. Several studies have identified populations of miRNAs that are associated with specific stem cell types. MicroRNAs mediate regulation of stem cell division,[79] as well as differentiation in adipocyte,[80] cardiac,[81] neural,[82] and hematopoietic lineages.[83] By comparing the miRNA expression profile of hemangiomas to normal endothelial cell controls we hope to describe the specific pathways involved in the pathogenesis of these lesions.

Mechanisms Controlling Hemangioma Growth – piRNA Regulation

Assuming that hemangiomas of infancy represent tumors of stem cell origin it will be important to identify other potential regulators of the growth and clinical behavior of these lesions. Previous work by Narayan's lab identified that hemangiomas produce BORIS, a pathologic protein that is normally only expressed in the testes. BORIS is a

member of the cancer-testis (CT) gene family that comprises genes normally expressed only in the testis, but abnormally activated in different malignancies. Its normal role in the testis consists of resetting genome-wide DNA methylation during spermatogenesis.[84] Hemangiomas, however, are the first benign tumor discovered to express this protein.

Given this localization of BORIS to the testis we hypothesized that other testis-specific regulatory proteins could be expressed in hemangiomas. Piwi proteins, a subclass of the family of Argonaute proteins, are expressed in germline and stem cells and they are associated with a unique set of small RNAs called piRNAs.

Recently discovered piRNAs (Piwi-interacting RNAs) represent an additional class of small non-coding RNAs distinct from microRNAs. This class of small RNAs differs from small interfering RNA (siRNAs) and microRNAs (miRNAs) in several important ways. [85-88] First, piRNAs interact with the Piwi, but not the Argonaute (Ago) subfamily of the Piwi-Ago family proteins. Second, piRNAs are 24 to 31 nucleotides instead of ~21. Third, piRNAs consist of more than 50,000 different species in contrast to hundreds of miRNAs. Fourth, these piRNAs are produced from long single-stranded precursors in contrast to siRNAs and miRNAs that are derived from double-stranded and short-hairpin RNA precursors, respectively.

Most piRNAs map to unique sites in the genome, including intergenic, intronic, and exonic sequences. For this reason piRNAs may have diverse functions including epigenetic programming, repressing transposition, and post-transcriptional regulation. Each of these speculated roles is supported by the known function of the partner Piwi proteins. For instance, Piwi is an epigenetic regulator.[89] It co-localizes with Polycomb group (PcG) proteins to cluster PcG response sequences in the genome.[90] It is thus

possible that Piwi-associated piRNAs may be involved in epigenetic regulation. In addition, Piwi prevents retrotransposon transposition in the testicular germ line, which suggests a second role of Piwi-associated piRNAs.[91]

The possible role of Piwi-associated piRNAs in the germline and in maintenance of stem cell pluripotency is what led us to further investigate the role of these RNAs in hemangiomas. We hypothesize that hemangiomas would express a unique pattern of piRNAs, the classification of which would provide further insight into the origin and pathogenesis of these lesions.

Growth of Hemangioma in Culture

One of the biggest limitations to hemangioma research has been the lack of a viable animal model. Currently available experimental models of hemangiomas include induction in mice via transgenic endothelial cells,[92] mouse endothelial cells that have been transformed by polyoma middle T oncogene,[93,94] and endothelial cells obtained from murine and human hemangiomas.[95] However, tumors generated from these cell lines have more malignant characteristics, are not obligately perinatal, and do not spontaneously regress.

An *ex vivo* culture system has been developed that uses intact human hemangioma tissue and permits biochemical manipulation.[96] However, while this model does produce microvascular outgrowths in culture, the hemangioma endothelial cells are not GLUT-1 positive.

Additional models which use purified cultures of hemangioma-derived cells have been used to demonstrate the unusual behavior of hemangiomas in terms of growth and

migration,[29,97] but these models lack the environmental factors found *in vivo* as potentially important growth factors, components of extracellular matrix, and direct influence from surrounding cells are not present. Drawbacks of this model also include a loss of cellular features of hemangioma endothelial cells and GLUT-1 expression.

It is clear that hemangiomas involve a population of both endothelial cells and multi-potent mesenchymal stems cells of perivascular origin. It seems that the interaction between these two cell populations leads to the growth of hemangiomas, and a successful model of hemangiomas will need to incorporate this synergistic effect.

Also of relevance to the development of a hemangioma model is the growth response of hemangiomas to estrogen. One group was able to demonstrate the response of hemangioma vascular endothelial cells to estrogen *in vitro*.[98] Elevated levels of 17-β estradiol[99] had previously been reported in children with hemangiomas. Xiao et al. demonstrated that the combination of 17-β estradiol and endothelial cell growth supplement in medium had a dramatic effect on proliferation of hemangioma endothelial cells in culture. The GLUT-1 positivity of these cells, however, was not verified. We hypothesize that if hemangiomas represent a population of mesenchymal stem cell tumors that culture media for stem cells would lead to *in vitro* proliferation of these cells.

Methods:

Specimen Collection

(Performed by the student)

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. 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. Human dermal microvascular endothelial cells (HDMEC), human umbilical vein endothelial cells (HUVEC), human adult endothelial cells, and neonatal endothelial cells were used as controls.

RNA preservation and extraction

(Performed by the student)

Immediately following tissue resection, 100-500mg of tissue was minced in 10ml of Qiagen 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 Celsius overnight and then frozen at -80 degrees Celsius until 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 with a mortar and pestle. All implements were baked at 200 degrees Celsius 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 ensure 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 isopropyl alcohol precipitation and ethanol washing steps, the pellets were allowed to dry for 15 to 30 minutes and were re-suspended in nuclease free water and stored at -80 degrees Celsius. To remove potential genomic contamination, 10µg 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 1 µl 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 quality control each sample was converted

into cDNA using the ABI 4368813 cDNA archive kit. All samples were then stored at -80 degrees Celsius.

5' end-labeling of RNA for piRNA detection

(Performed by the student)

Dephosphorylation of RNA

Shrimp alkaline phosphatase (SAP) dephosphorylation reaction was first performed on the RNA samples. The amount of sample needed for 2µg of RNA based on concentration was calculated. Next the amount of H_2O needed to bring the reaction volume up to 16.5µL was calculated. A master reaction mix of 10X SAP buffer, RNase Out, and SAP was made. A reaction mix of 3.5µL/tube was added for each sample (leaving 16.5µL for RNA and H_2O). Once the reaction mix, RNA, and H_2O were added to the tubes the reaction tubes were placed at 37˚C for 1 hour and then at 65˚C for 15 minutes in order to inactivate the SAP. The tubes were then stored at -80˚C.

5' end-labeling of RNA

A master mix for the end labeling reaction was made from 30µL of 10x T4PNK buffer, 5µL of RNase Out, 10µL of T4PNK (15U), and 55µL of H_2O. Then 10µL of master mix was added to each reaction tube from the dephosphorylation step bringing the total volume of each reaction tube to 30µL. The reaction tubes were then incubated at 37˚C for 1 hour. Next 1.5µL of 0.5M EDTA was added to each reaction tube and the tubes were incubated at 65˚C for 15 minutes. The unincorporated 32-P nucleotides were removed by spinning the samples through Roche G25 mini quick oligo spin columns. The

Roche oligo columns were prepared in the following fashion: First the columns were allowed to warm to room temperature. The top cap was removed from each column and then the bottom tip was snapped off. The columns were placed in Eppendorf tubes and then spun at 1200x for 1 minute. The columns were then placed in clean, labeled Eppendorf tubes and the labeled samples were pipetted directly onto the middle of the matrix. The columns were then spun at 1200x for 4 minutes in order to collect the labeled sample in the flow-through. The columns with the unincorporated nucleotides were then discarded into a closed container in order to keep the 'hot' column matrix contained. The labeled flow-through was then confirmed with a Geiger counter. To each sample tube 3µL of 6x load dye and formamide was added and the tubes were then stored at -20˚C.

Running Labeled RNA on denaturing poly-acrylamide gel

A 15% 6M urea poly-acrylamide gel was made by combining 13.125mL of 40% acrylamide:Bis (19:1), 12.54g urea, 3.5mL of 10x TBE and DEPC H_2O to bring the final reaction mixture volume to 35mL. To this 35mL mixture was added 350µL of 10% APS and 35µL TEMED (in order to polymerize the gel). The gel was then poured between two cleaned glass plates and was allowed to sit for 30 minutes in order to polymerize. The gel was pre-run in 1x TBE in H_2O_{DEPC} for 15 minutes at 230V. The wells were flushed occasionally to get rid of urea. After a pre-run was completed the samples were loaded into the gel and were run at 230V for 2 hours (until the dye front was just above the bottom of the gel). After completion the gel was visualized by phosphoimager on x-ray film.

Quantitative RT-PCR for CTCF, BORIS, H19, IGF-2, Oct-4, C-myc, Nanog, Sox-2, DLK, p16, RB, and CDX-2

(Performed as a service by Center for Functional Genomics, Albany, NY)

Nineteen hemangioma samples (4 proliferative, 10 quiescent, 5 involuting) and 4 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. RNA was converted to first strand cDNA by use of the RETROscript First Strand Synthesis Kit (Ambion, Austin, TX) and primed with oligo-dT according to the manufacturers specifications. Taqman gene expression assays to quantify gene expression were obtained from Applied Biosystems (Foster City, CA), and are identified below.

Assay ID:	Gene Name:
Hs00902008_m1	CTCF
Hs01005963_m1	IGF2
Hs00540744_m1	CTCF-L (BORIS)
Hs00895526_m1	H19
Hs00905030_m1	C-myc
Hs02387400_g1	Nanog
Hs01053049_s1	Sox2
Hs03005111_g1	Oct4
Hs00171584_m1	DLK
Hs99999189_m1	p16
Hs01078066_m1	RB
Hs01078080_m1	CDX-2
Hs99999905_m1	GAPDH

Two-step Taqman-based quantitative RT-PCR was performed. First strand cDNA was synthesized as described above, and the cDNA equivalent of 20 ng starting RNA was then included as template in qPCR reactions. QPCR Reactions to detect gene expression contained 1X Taqman Master Mix (Applied Biosystems) and 1X gene-specific assay

reagents as recommended by the manufacturer (Applied Biosystems). All reactions were run in triplicate. Reactions that did not contain template cDNA were included as negative controls

Reaction plates were processed on an Applied Biosystems 7900HT Sequence Detection System. The AmpliTaq Gold polymerase was activated at 95^{o}C for 10 min followed by 40 cycles consisting of denaturation for 15 seconds at 95^{o}C and annealing and extension for 60 seconds at 60^{o}C.

Amplification data was analyzed with the ABI Prism SDS 2.1 software (Applied Biosystems). Relative quantification of gene expression was performed by the ΔΔCt method.[100] GAPDH expression served as an endogenous control to normalize expression within each sample.

The student performed data analysis in collaboration with the Yale University Department of Biostatistics. The nineteen cases were compared with the four controls using a two-sample, two tailed, t-test, assuming unequal variances. The p-values were ranked and compared with the Bonferroni correction for multiple comparisons. Specifically, the p-values need to attain a level of significance was determined by the following calculation: 0.05 / 22 = .00227, where 22 equals the number of comparisons.

MicroRNA Microarray

(Performed as a service by LC Sciences, Houston, TX)

This experiment consisted of nine hemangioma samples ages: 81, 165, 286, 299, 380, 590, 752, 1171, and 3626 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: adult and neonatal 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 were subjected to unbiased cluster analysis.

The assay started from 2 to 5 µg total RNA sample, which was size fractionated using a YM-100 Microcon centrifugal filter (from Millipore) and 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; two different tags were used for the two RNA samples in dual-sample experiments. 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 target microRNA (from miRBase, 11.0 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 (photogenerated reagent) chemistry. The hybridization melting temperatures were balanced by chemical modifications of the detection probes. Hybridization used 100 µL 6xSSPE buffer (0.90 M NaCl, 60 mM Na_2HPO_4, 6 mM EDTA, pH 6.8) containing 25% formamide at 34 °C. After hybridization, detection used fluorescence labeling, using tag-specific Cy3 and Cy5 dyes. Hybridization images were collected using a laser scanner (GenePix 4000B, Molecular Device) and digitized using Array-Pro image analysis software (Media Cybernetics). Data was analyzed by first subtracting the background and then

normalizing the signals using a LOWESS filter (Locally-weighted Regression). For two color experiments, the ratio of the two sets of detected signals (log2 transformed, balanced) and p-values of the t-test were calculated; differentially detected signals were those with less than 0.01 p-values.

Additional data analysis was performed at Yale University in the Department of Biostatistics. The nine cases were compared with the two controls using a two-sample, two tailed, t-test, assuming unequal variances. The p-values were ranked and compared with the Bonferroni correction for multiple comparisons. Specifically, the p-values need to attain a level of significance was determined by the following calculation: 0.05 / 886 = .000056, where 886 equals the number of markers examined.

microRNA Pathway Analysis Using Ingenuity Software and MetaCore Software (Performed as a service by Bioinformatics at Yale)

Data was first analyzed through the use of Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com).

1. Network Generation

A data set containing gene identifiers and corresponding expression values was uploaded into the application. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. A 1.5-fold expression threshold, and p-value <0.05 was set to identify genes whose expression was significantly differentially regulated. These genes, called focus genes, were overlaid onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge

Base. Networks of these focus genes were then algorithmically generated based on their connectivity.

2. Functional Analysis of an Entire Data Set

The Functional Analysis identified the biological functions and/or diseases that were most significant to the data set. Genes from the dataset that met the 1.5 fold expression threshold, and p-value <0.05 and were associated with biological functions and/or diseases in the Ingenuity Pathways Knowledge Base were considered for the analysis. Fischer's exact test was used to calculate a p-value determining the probability that each biological function and/or disease assigned to that data set is due to chance alone.

3. Functional Analysis of a Network

The Functional Analysis of a network identified the biological functions and/or diseases that were most significant to the genes in the network. The network genes associated with biological functions and/or diseases in the Ingenuity Pathways Knowledge Base were considered for the analysis. Fischer's exact test was used to calculate a p-value determining the probability that each biological function and/or disease assigned to that network is due to chance alone.

4. Canonical Pathway Analysis: Entire Data Set

Canonical pathways analysis identified the pathways from the Ingenuity Pathways Analysis library of canonical pathways that were most significant to the data set. Genes

from the data set that met the 1.5 fold expression threshold, and p-value <0.05 and were associated with a canonical pathway in the Ingenuity Pathways Knowledge Base were considered for the analysis. The significance of the association between the data set and the canonical pathway was measured in 2 ways: 1) A ratio of the number of genes from the data set that map to the pathway divided by the total number of genes that map to the canonical pathway is displayed. 2) Fischer's exact test was used to calculate a p-value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone.

5. Network/My Pathways Graphical Representation

A network/My Pathways is a graphical representation of the molecular relationships between genes/gene products (See Image 15). Genes or gene products are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). All edges are supported by at least 1 reference from the literature, from a textbook, or from canonical information stored in the Ingenuity Pathways Knowledge Base. Human, mouse, and rat orthologs of a gene are stored as separate objects in the Ingenuity Pathways Knowledge Base, but are represented as a single node in the network. The intensity of the node color indicates the degree of up- (red) or down- (green) regulation. Nodes are displayed using various shapes that represent the functional class of the gene product.

Data was also analyzed using MetaCore™ software version 5.2 from GeneGo Inc.

MetaCore™ is an integrated software suite for functional analysis of experimental data. The scope of data types includes microarray and SAGE gene expression, SNPs and CGH arrays, proteomics, metabolomics, pathway analysis, Y2H and other custom interactions. MetaCore™ is based on a proprietary manually curated database of human protein-protein, protein-DNA and protein-compound interactions, metabolic and signaling pathways and the effects of bioactive molecules in gene expression. MetaCore™ uses 10 different network-generating algorithms and includes specificity filters for tissues, functional processes, sub-cellular localization, interaction mechanisms, and species.

Hemangioma Cell Culture

(Performed by the student)

Freshly resected hemangioma tissue was minced into 1mm cubes. A portion of the sample was placed in collagenase and incubated at 37˚ C in 5% CO_2 / 95% air for 20 minutes following mincing. Tissue sample not degraded by collagenase was divided equally into three parts. One portion was suspended in 100mL modified TeSR1 basal medium for maintenance of human embryonic stem cells (mTeSR1, StemCell Technologies). A second portion was suspended in 100mL mTeSR1 with the addition of 20mL of 5x supplement (StemCell Technologies) containing recombinant human basic fibroblast growth factor (rhbFGF) and recombinant human transforming growth factor β (rhTGF β). A third portion was suspended in 100mL mTeSR1 with the addition of 20mL

of 5x supplement, 10μL of basic fibroblast growth factor (bFGF, StemCell Technologies) with a concentration of 0.500 mg/mL, and 50μL of heparin (concentration 100ng/mL). These samples were then plated on a 12-well culture plate, 4 wells per sample, and incubated overnight at 37˚ C. The sample treated with collagenase was then divided into three portions as described above. One mL of each of the three collagenase samples, and 1 mL of each of the three non-collagenase samples were then plated on a six-well BioFlex plate containing collagen IV membrane (Flexcell International Corp.) This was then repeated on a six-well BioFlex plate containing elastin membrane (Flexcell International Corp).

Additional tissue sample was cultured according to the method published by Xiao et al.[98] Tissue specimen was placed in standard culture medium consisting of Medium 199 (M-199; Gibco, Rockville, MD) supplemented with 200μg/mL streptomycin, 75μg/mL endothelial cell growth supplement (ECGS; Sigma, St. Louis, MO) and 50pg/mL of 17β-estradiol (Sigma).

Summary of Methods:

Performed by Student:

- Specimen Collection
- RNA extraction
- piRNA detection
- Hemangioma cell culture

Performed as Services:

- Quantitative RT-PCR of IGF-2, H19, BORIS, CTCF, DLK, Oct4, Sox2, Nanog, C-myc, CDX-2 (Center for Functional Genomics)
- microRNA microarray (LC Sciences)
- microRNA pathway analysis using Ingenuity Software and MetaCore Software (Bioinformatics at Yale)

Additional Experiments to Complete:

This thesis represents a work in progress and additional experiments await completion in order to continue to test the hypothesis that hemangiomas represent mesenchymal stem cell tumors derived from pericytes. A list of anticipated experiments is below with appropriate explanation in the discussion section:

- Confirm expression of Oct-4, C-myc, Sox-2, and Nanog with western blot
- Examine hemangiomas for expression of nestin and sox10 (markers of neural crest derived cells)
- Perform quantitative RT-PCR on samples for DLK, PDGFR-2, and NG2, PPAR-γ
- Using a lentivirus vector treat hemangioma cell cultures with siRNA to DLK to show differentiation to adipocytes
- Identify single nucleotide polymorphisms (SNPs) at the DLK gene locus. Treat hemangioma cell cultures with thiazolidinediones (a class of anti-diabetic drugs that are PPAR-γ receptor ligands and induce adipocyte differentiation) and measure time to conversion from hemangioma tissue to adipose.

Results:

Quantitative RT-PCR to Determine Expression Levels of: IGF-2, H19, CTCF, BORIS, Oct-4, C-myc, Sox-2, Nanog, RB, p16, DLK, and CDX-2

We examined the transcription factors of "stemness" Oct-4, C-myc, Sox-2 and Nanog in 19 hemangiomas (representing all three stages of growth) compared to control adult endothelial cells, neonatal endothelial cells, human dermal endothelial cells and human umbilical vein endothelial cells by quantitative RT-PCR. We found that all hemangiomas expressed factors Oct-4, Sox-2, C-myc, and Nanog at levels higher than endothelial cell controls (See Image 7). Taken individually these differences achieve statistical significance with p-values < 0.05; (See Image 8). Because multiple comparisons were made, this must be taken into account using the Bonferroni correction ($0.05/22 = 0.00227$). The new threshold for significance is 0.002, and none of the stem cell transcription factors reach this corrected threshold for significance.

In addition to transcription factors known to be upregulated in embryonic stem cells, we examined several genes implicated in the growth and pathogenesis of hemangiomas. These genes included IGF-2 (mitogenic, pro-angiogenic, and growth promoting), CTCF (universal transcription factor and regulator of IGF-2), RB (tumor suppressor), p16 (tumor suppressor), and DLK (negative regulator of adipogenesis and adipocyte maturation).

Our results confirm the increased IGF-2 produced by hemangiomas at a statistically significant level of $p < 0.001$. IGF-2 is a pro-angiogenic and mitogenic factor that has been implicated in the growth of hemangiomas.[46] The limited number of samples

we examined and the variability in IGF-2 production, however, prevented us from differentiating the level of IGF-2 production between proliferating, plateau, and involuting hemangioma samples. Our results also indicate that CTCF production is increased by a factor of 4 in hemangiomas compared to controls (p-value <0.002). This transcription factor binds to the IGF-2 locus and it is believed that increased levels of this transcription factor lead to decreased levels of IGF-2.[64] The transcription factor BORIS was detectable, although at very low levels, in ten of nineteen samples.

RB is a widely expressed tumor suppressor protein that is dysfunctional in many types of cancer. An initial hypothesis predicted that this protein would be downregulated in hemangiomas given its role in arrest of the cell cycle. Surprisingly, the expression of the tumor suppressor gene retinoblastoma (RB) was increased in hemangioma samples by a factor of three compared to endothelial cell controls; and, this result was statistically significant at the $p < 0.001$ level.

DLK is significant in that it represents a critical protein that is involved in the control of adipocyte differentiation of pre-adipocytes. A mere two-fold increase in expression of DLK was found to robustly prevent the conversion of 3LT3 pre-adipocytes to adipocytes.[36] A previous study[46] demonstrated a decrease in DLK expression by five fold during the hemangioma maturation process. The variability between our samples was too great to detect a significant decrease in DLK expression during the hemangioma maturation process. However, our results indicate that hemangiomas express DLK at a level greater than 10^5 orders of magnitude compared to controls (See Image 7) and this result is statistically significant at the $p < 0.001$ level. Thus, the very high levels of DLK,

especially in the proliferating hemangiomas, argue for a role of this gene in the suppression of fat conversion.

We also examined hemangioma samples for expression of CDX-2, a gene normally expressed by the placenta. Previous studies have shown that CDX-2 is sufficient to induce differentiation of embryonic stem cells down a trophoectoderm lineage, which eventually forms the placenta. Furthermore, reciprocal inhibition between CDX-2 in trophoectoderm and Oct3/4 in pluripotent cells is thought to be involved in segregation of these cell lineages.[101] We were unable, however, to detect the placental specific transcription factor CDX-2 in any of our samples.

PiRNA Analysis

PiRNA analysis revealed that hemangiomas do not contain piRNA transcripts, thus excluding the role of these molecules in regulation of hemangioma growth and pathogenesis. Image 3 shows the absence of a piRNA band in all 7 hemangioma specimens as well as the control sample of HUVEC.

MicroRNA Microarray Analysis

Data analysis generated a list of microRNAs expressed in hemangiomas at a level statistically significant from the controls (adult and neonatal HDMEC). This list is displayed in Image 9. In addition, microRNA profiles of the 9 samples and 2 controls were clustered (average linkage, correlation similarity). The results of this analysis are displayed in Images 11 and 12. However, when the microRNA expression profiles of the three proliferating hemangioma samples were compared to the three involuting samples

there was no statistically significant difference in expression profiles. The reason for this may be attributable to our small sample size and the large number of microRNAs analyzed. Given the large number of comparisons made between specimens and controls, results required a p-value of < 0.000056 in order to attain significance, when using the Bonferroni correction (0.05/886 = 0.000056).

Despite this high threshold for significance, our microarray data indicates that microRNAs associated with mesenchymal stem cells (miR-143, miR320a, miR320c, let-7c) are upregulated in hemangiomas compared to controls. Of particular interest, miR-143 was significantly upregulated in this group of tumors by over 100x compared to controls ($p< 0.000056$). Increased levels of miR-143 have been observed in differentiating adipocytes, and inhibition of miR-143 has been shown to effectively inhibit adipocyte differentiation.[80] MiRNA 195, a microRNA known to target DLK, was also upregulated more than 25x in hemangioma samples.

Both let-7c and let-7g have increased expression levels in hemangiomas compared to controls. Let-7g is upregulated 2x and let-7c is upregulated 1.5x in hemangiomas compared to controls ($p<0.000056$). Let-7 was originally identified in *C. elegans* in a screen seeking genes that regulate developmental timing.[102] Loss of function of this miRNA prevents normal transition of late larval to adult cell fates. There are 12 let-7 homologs in the human genome, organized in eight distinct clusters. At least four of these clusters are contained within regions known to be frequently deleted in diverse human malignancies.[103] Furthermore, two studies have documented frequent downregulation of let-7 family members in lung cancer associated with poor prognosis.[104, 105]

Analysis of hemangioma specimens also revealed decreased expression of miR-106a by 2x compared to controls. The retinoblastoma (RB1) 3'UTR is found to be functionally interactive with miR-106a.[106] The biological significance of this proven miRNA::mRNA interaction is reinforced by previous reports showing that RB1 gene is normally transcribed in colon cancers whereas various fractions of cells do not express RB1 protein.[107] This finding suggests a post-transcriptional mechanism for regulation of RB1, which could possibly be explained by the concomitant overexpression of miR-106a detected in colon carcinoma.[106]

MicroRNA Pathway Analysis

Ingenuity software helped identify key molecules and pathways that could potentially play a role in the pathogenesis of hemangiomas. Of 603 microRNAs in the initial list 466 were recognized by Ingenuity and 150 were eligible for network analysis. With a 1.5-fold expression threshold and p-value <0.05, 65 miRNAs were network analysis eligible. The top biological functions implicated in hemangioma pathogenesis were cancer and reproductive system disease. Both the cardiovascular and nervous systems were implicated as developmental systems involved in hemangioma pathogenesis. A complete list is shown in Image 13.

When these biological functions are organized into networks and then ranked according to the –log of the p-value, the network that includes cancer, reproductive system disease, cellular growth and proliferation is most significantly involved. A complete list of the networks implicated in hemangioma pathogenesis is shown in Image 14.

The Cancer, Reproductive System Disease, Cellular Growth and Proliferation Network is displayed in the appendix (See Image 15). In-depth analysis of these networks falls outside the scope of our hypothesis because the vast majority of transcription factors and miRNAs pictured have yet to be directly linked to hemangiomas. However, this image is included to demonstrate the data generated by this analysis and to show how the creation of such networks can elucidate the transcription factors and second messengers that function as downstream targets of the miRNAs differentially expressed in hemangiomas. The identification of key pathways involved in hemangioma pathogenesis will prove to be important in developing new therapeutics that more specifically target hemangiomas than the ubiquitously prescribed corticosteroids that now comprise the mainstay of treatment for these lesions.

Pathway analysis was also performed using MetaCore software and the results of this analysis are more directly relevant to evaluation of our hypothesis that hemangiomas represent tumors of mesenchymal stem cell origin. The MetaCore software generates networks centered on specific transcription factors and suggests which transcription factors regulate the indicated genes. Members of the target list are shown with a blue or red circle next to their icons, where red indicates upregulation and blue indicates downregulation in the experiment. Red lines indicate inhibition, and green lines activation, as indicated from the literature.

Image 16 shows the relationship between C-myc and numerous miRNAs. Of the miRNAs pictured only let-7c and let-7a-1 regulate C-myc expression, and both have an inhibitory effect. The rest of miRNAs depicted are downstream targets of C-myc. Similar images are generated for p53 and Sox2 (See Image 17). These images show how the

differentially expressed microRNAs in hemangiomas interact with various other transcription factors.

Another function of MetaCore allows for Interactome analysis, which was performed with the miRNA genes that were 1.5x upregulated (at $p<0.05$), and combined with genes implicated in hemangioma pathogenesis. This analysis begins by identifying genes found in the literature or reported in the MetaCore disease database as associated with hemangiomas (See Image 18). These genes were then added to the miRNA gene set to strengthen the network analysis.

The Interactome analysis table (See Image 19) shows transcription factors having a significantly high number of outgoing connections (i.e., connections of the type "regulates") to members of the target list. The False Discovery Rate parameter for the list was set at 0.05 (i.e. 1 out of 20 are likely false). Those transcription factors that have a small number of connections (like r=1) to list members are the least reliable ones.

Image 21 shows a network of miRNAs and known hemangioma related genes. This is most useful image in highlighting the key molecules involved in the regulation of hemangioma-related genes. The threshold for differential expression of miRNA is set to 4x in order to increase the specificity of the pathways highlighted. Image 22 highlights the shortest pathway between hemangioma related genes and differentially expressed miRNAs.

MetaCore then uses the list of differentially expressed miRNAs and a list of known hemangioma genes derived from the scientific literature to develop a list of disease processes that share biomarkers with hemangiomas. The results of this analysis are displayed in Images 23 and 24. Image 23 uses only the list of differentially expressed

miRNAs, while Image 24 combines these miRNAs with known hemangioma related genes.

Similarly to Ingenuity, MetaCore identified uterine neoplasms and disease as the processes sharing most pathways and biomarkers with hemangiomas. Image 25 gives a graphical representation of the pathways shared by hemangiomas and uterine disease and the key players in these pathways. As highlighted miR-21 and let-7a-1 are both involved as well as the transcription factors CTCF, BORIS, and C-myc, all of which exert influence on IGF-2, a key regulator of hemangioma proliferation and growth.

Hemangioma Cell Culture

None of the hemangioma tissue specimens placed in embryonic stem cell medium demonstrated growth or vascular sprouting in culture after incubation for seven days. The hemangioma tissue that was placed in standard culture medium supplemented with 200μg/mL streptomycin, 75μg/mL endothelial cell growth supplement demonstrated growth at the one-week mark. Growth became prominent at the 2-½ week mark.

Summary of Results:

- Hemangiomas express the stem cell related transcription factors Oct-4, Sox-2, C-myc, and Nanog at levels higher than endothelial cell controls. This is significant at the $p < 0.05$ level but not at the 0.002 level required by the Bonferroni correction for multiple comparisons.

- Hemangiomas express IGF-2, RB, and DLK at a level higher than endothelial cell controls ($p < 0.001$).

- Hemangiomas do not express the placental transcription factor CDX-2.

- Hemangiomas do not contain piRNA transcripts.

- Hemangiomas express the following miRNAs (miR-143, miR320a, miR320c, let-7c) that are associated with mesenchymal stem cells at levels higher than controls ($p < 0.000056$).

- miR-143 is upregulated in hemangiomas more than 100x compared to controls ($p < 0.000056$). Increased levels of miR-143 have been observed in differentiating adipocytes and inhibition of miR-143 has been shown to effectively inhibit adipocyte differentiation.

- miR-195, known to target DLK, is upregulated more than 25x compared to controls ($p < 0.000056$).

- Embryonic stem cell media supplemented with recombinant human basic fibroblast growth factor (rhbFGF) and recombinant human transforming growth factor β (rhTGF-β) did not lead to vascular sprouting and culture of hemangioma tissue.

Discussion:

Hemangioma Placenta Connection

While it is no longer believed that hemangiomas represent embolized placental tissue, the similarities between the two tissues should help us think about hemangiomas as tumors composed of cells performing a barrier function. Just because hemangiomas are not placental tissue does not mean that we should abandon investigation process into the overlap between them. Two independent microRNA pathway analysis programs, Ingenuity and MetaCore, identified reproductive system disease (Uterine Disease in MetaCore) as an important network regulated by microRNAs differentially expressed by hemangiomas (See Images 15 and 25). MicroRNA 21 is predicted to be a critical player in both of these networks, and microarray analysis shows upregulation of this molecule in hemangiomas of all stages of growth.

A potential oncogenic role for miR-21 was first discovered in a screen for abnormally expressed miRNAs in glioblastoma.[108] Using an expression array and northern blotting, miR-21 was demonstrated to be consistently upregulated in human glioblastoma tumor tissues, primary tumor cultures, and established glioblastoma cell lines relative to normal fetal and adult brain tissue and primary cultured neurons and astrocytes. Knockdown of miR-21 in glioblastoma cell lines led to activation of caspases and a corresponding induction of apoptotic cell death. Support for a more general role of miR-21 in cancer was recently observed with widespread overexpression of miR-21 in diverse tumors including those from the breast, colon, lung, pancreas, stomach, and prostate.[106]

Our research has uncovered further differences between placental tissue and hemangiomas with the fact that hemangiomas do no express the placental specific transcription factor CDX-2. This transcription factor is involved in the differentiation of embryonic stem cells down a trophoectoderm lineage,[101] which eventually develops into the placenta. The question still remains why do hemangiomas share so many markers with placental tissue? The answer to this may lie in the fact that placental tissue contains a large population of pericytes.

Additional connections between placental tissue and hemangiomas remained to be discovered. Previous work by Narayan's lab identified that hemangiomas express the pathologic protein BORIS, which is normally only expressed in the testis. BORIS is a member of the cancer-testis (CT) gene family that comprises genes normally expressed only in testis, but abnormally activated in different malignancies. Its normal role in the testis consists of resetting genome-wide DNA methylation during spermatogenesis. Given this localization of BORIS to the testis, we hypothesized that other testis-specific regulatory proteins, specifically piRNAs, could be expressed in hemangiomas. However, our results indicate that piRNAs are not present in hemangiomas and therefore do not play a role in the growth or pathogenesis of these lesions.

Hemangiomas as Mesenchymal Stem Cell Tumors

It has long been thought that hemangiomas are endothelial cell tumors. One group has shown that endothelial progenitor cells are present in hemangiomas.[27] However, not all samples (eleven of twelve) demonstrated CD133+/CD34+ endothelial progenitor cells. Furthermore, no such endothelial progenitor cells were identified in involuting

hemangiomas. If hemangiomas were in fact endothelial cell tumors, one would expect *all* hemangioma specimens to contain endothelial progenitor cells, cells which are present at the start of the tumor and persist throughout the lifecycle of the tumor.

These studies examined hemangioma clonality using X-linked human androgen receptor gene assay (HUMARA) analysis. One study analyzed cells grown in culture from seven patients, and all showed a skewing toward a single allele.[29] A subsequent study analyzed intact tissue without selection of cells and found some degree of allelic loss in 12 of 14 samples.[30] These two independent investigations suggest that hemangiomas arise as a consequence of clonal expansion from a single cell, however, it is possible that sampling constraints biased these results. It is notable that 2 of the 14 samples did not show allelic loss, an argument against endothelial cell clonality in hemangiomas.

It was recently demonstrated that a population of stem cells exist within hemangiomas.[31] These cells, termed hemangioma mesenchymal stem cells (Hem-MSCs), were isolated from 14 proliferating and five involuting hemangiomas by taking advantage of mesenchymal stem cells' selective adhesion to plastic bacteriologic dishes.[32] These Hem-MSCs exhibited a random pattern of X-inactivation indicating that these cells are not clonally derived. Authors of this study hypothesized that these mesenchymal stem cells originated in the bone marrow and through selective homing arrived at the site of the developing hemangioma. A recent lineage study shows, however, that pericytes are the source of these mesenchymal stem cells.[35]

Even more intriguing is the possibility that the endothelial cells present in hemangiomas arise from mesenchymal stem cells. If this were the case, a single cell type

could explain the pathogenesis of these lesions. One group identified a population of cells (CD34+/CD31-/CD45-) from the stromovascular portion of adipose tissue that could differentiate into endothelial cells.[109] Another group found a similar population of cells (CD31-/CD34-/CD106-/VCAM-/fetal liver kinase+) from adipose tissue that expressed endothelial markers when cultured with VEGF.[110] Given that this population of mesenchymal stem cells can differentiate into endothelial cells the causative defect in hemangiomas may reside within this population of cells which then gives rise to a proliferation of endothelial cells.

Even if mesenchymal stem cells are not the source of cells that differentiate into endothelial cells, additional studies have shown that these multipotent mesenchymal stem cells of perivascular origin serve both structural and functional roles in interactions with endothelial cells.[33] Hemangiomas may proliferate as a result of the unique interaction between mesenchymal stem cells and endothelial cells in response to hypoxia in the local environment or other potential causes.

By using quantitative RT-PCR, we demonstrated that hemangiomas specimens express transcription factors and genes known to play a role in the maintenance of stem cell pluripotency at a level greater than control endothelial cells. Specifically, transcription factors Oct-4, Sox-2, C-myc, and Nanog are increased. This corroborates an earlier study that identified mesenchymal stem cells within hemangioma tissue that are distinct from endothelial progenitor cells.[31]

We chose to analyze transcription factors based on previous studies that identified Oct-4,[111,112] Sox-2,[113] C-myc, and Nanog[114] as factors that play a role in the maintenance of pluripotency in both early embryos and embryonic stem cells. These factors were

selected for analysis because Takahashi and Yamanaka demonstrated that adult fibroblast cells can be reprogrammed into pluripotent stem cells by the induction of four critical factors: Oct3/4, Sox2, C-myc, and Klf4.[112] In addition, Oct4 plays a critical role in the establishment and maintenance of pluripotent cells in a pluripotent state.[115]

Expression of Sox2, Oct-4, Nanog, and C-myc at levels higher than control endothelial cells does not confirm that hemangiomas represent tumors derived solely from mesenchymal stem cells. However, the expression of these factors does confirm that there is a population of stem cells within hemangioma tissue and gives further evidence to the role of this population of cells within hemangiomas.

A microRNA microarray lends further support to this hypothesis. A previous study[116] has identified a set of microRNAs associated with mesenchymal stem cells. Of these miRNAs our analysis indicates that miR-143, miR320a, miR320c, and let-7c (all identified in mesenchymal stem cells) are upregulated in hemangioma tissue in all stages of growth.

Given that a population of mesenchymal stem cells (SH2+/CD105+/SH3+/SH4+/CD90+/CD29+/smooth muscle α–actin+/CD133+/CD45-/CD14-/CD34-/CD31-)* exists within hemangiomas, it is important to better characterize the nature of these cells. The first step towards this will be identifying the specific cell markers of this population of cells. To date the best method for identifying the pluripotent stem cell population found in the stromovascular portion of adipose tissue is using the profile: CD34+/CD31-/CD45-/CD144-, and 95% of this population of cells co-express mesenchymal cell markers (CD10, CD13, CD90) and surface markers used to identify

* The CD34 positivity of this population of cells is uncertain because it has been shown that CD34+ is downregulated in culture (117).

pericytes (NG2 and PDGFR).[33] In future experiments it will be helpful to try and isolate this specific population of cells from hemangiomas.

Pericytes are the origin of mesenchymal stem cells

We are just beginning to understand the relationship between pericytes and mesenchymal stem cells. Recently, it has been demonstrated that pericytes are the population of cells that give rise to mesenchymal stem cells that then develop into adipocytes.[35] It stands to reason that if hemangiomas are mesenchymal stem cell tumors, then they have likely developed from pericyte origins. At the time of completion of this thesis experiments to examine the recognized markers of pericytes (PDGFR and NG2) in hemangioma specimens are pending. Hemangioma pathologic specimens will be stained for PDGFR and NG2 as well.

Pericytes of the head and neck are derived from neural crest while pericytes in the rest of the body derive from mesoderm. In order to investigate the potential neural crest origins of hemangiomas we plan to analyze samples for nestin and sox10, which are considered to be the best markers of neural crest-derived cells.

Regulation of Adipogenesis in Hemangiomas

As we begin to develop an understanding of the role of mesenchymal stem cells in hemangiomas, it is important to identify those genes responsible for the progression and development of the tumor. One gene that holds great promise is DLK, also known as Pref-1. DLK is a cell surface protein in the epidermal growth factor family[36] that

functions as a negative regulator of adipocyte differentiation. A previous study has shown that DLK is decreased five fold during the hemangioma maturation process.[46] Our results confirmed that DLK is increased in hemangiomas compared to controls, but there was too much variability between groups to say with certainty whether DLK expression is decreased in involuting hemangiomas compared to proliferating specimens.

The results of our microRNA microarray also show that miR-143 is increased in hemangiomas compared to controls. This is of particular interest because increased levels of miR-143 were observed in differentiating adipocytes, and inhibition of miR-143 has been shown to effectively inhibit adipocyte differentiation.[80]

DLK may be a potential therapeutic target in the treatment of hemangiomas. Downregulation of DLK, possibly through the use of siRNA, could hasten the development of hemangiomas into fibroadipose tissue. In order to test this hypothesis we plan to treat hemangioma cell cultures with siRNA to DLK using a lentivirus vector, to determine if this speeds progression of cells into adipose tissue.

Along these lines of investigation we also plan to identify single nucleotide polymorphisms at the DLK gene locus. Freshly resected hemangioma specimens will be genotyped for these polymorphisms and then grown in culture. Once in culture, samples will be treated with thiazolidinediones, a class of anti-diabetic drugs that are peroxisome proliferator-activated receptor γ (PPAR-γ) ligands that induce adipocyte differentiation and increase the expression of PPAR-γ protein. We will then measure time to conversion from hemangioma tissue to adipose and correlate that with DLK polymorphisms.

Conclusion:

The results of our investigation do not conclusively implicate mesenchymal stem cells as the cells of origin of hemangiomas. However, we have demonstrated that hemangiomas express genes involved in the maintenance of stem cell pluripotency and express microRNAs transcripts that are markers of mesenchymal stem cells at a level significantly greater than endothelial cell controls. Important pieces of evidence are still necessary to prove or disprove our hypothesis and several of these experiments are pending. We must demonstrate that hemangiomas express markers of pericytes (NG2, PDGFR-β). We must demonstrate that hemangiomas on the head and neck are of a neural crest origin. Most importantly we must isolate the implicated cells of origin and show that they can reconstitute hemangiomas in a culture-based model.

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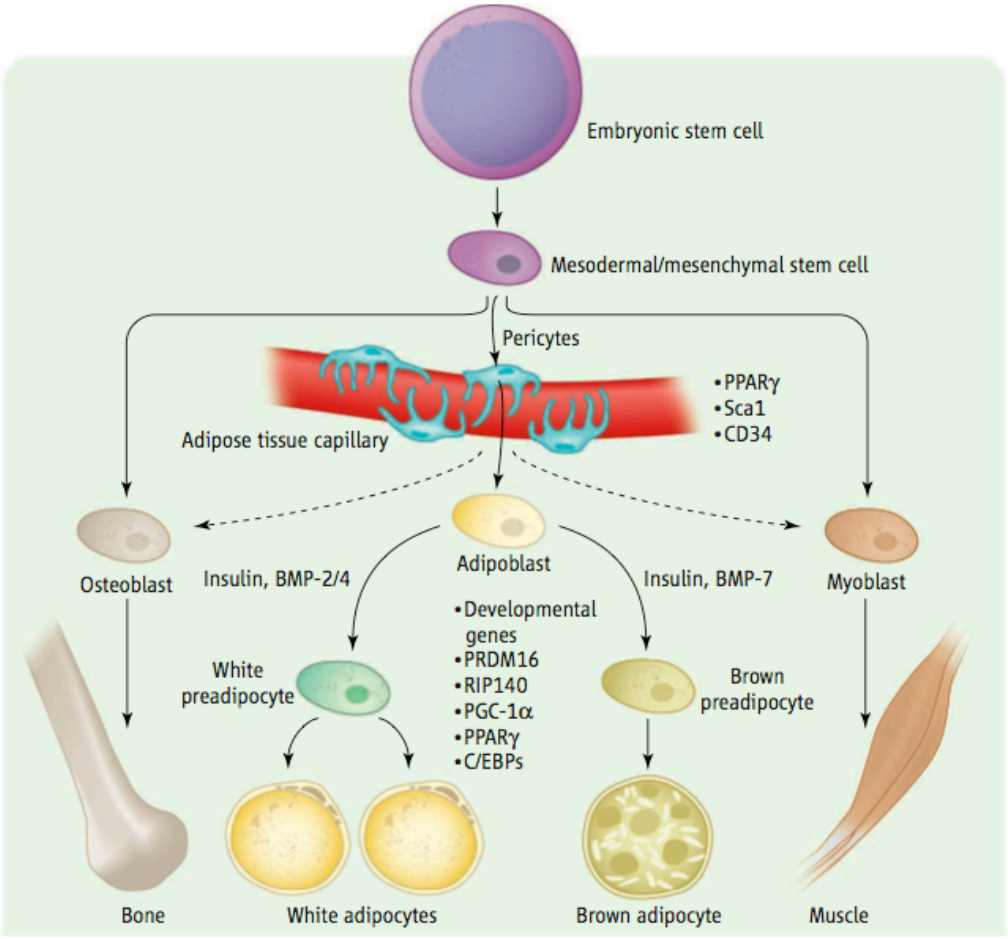
Hypothesized Perivascular Origin of Adipocytes



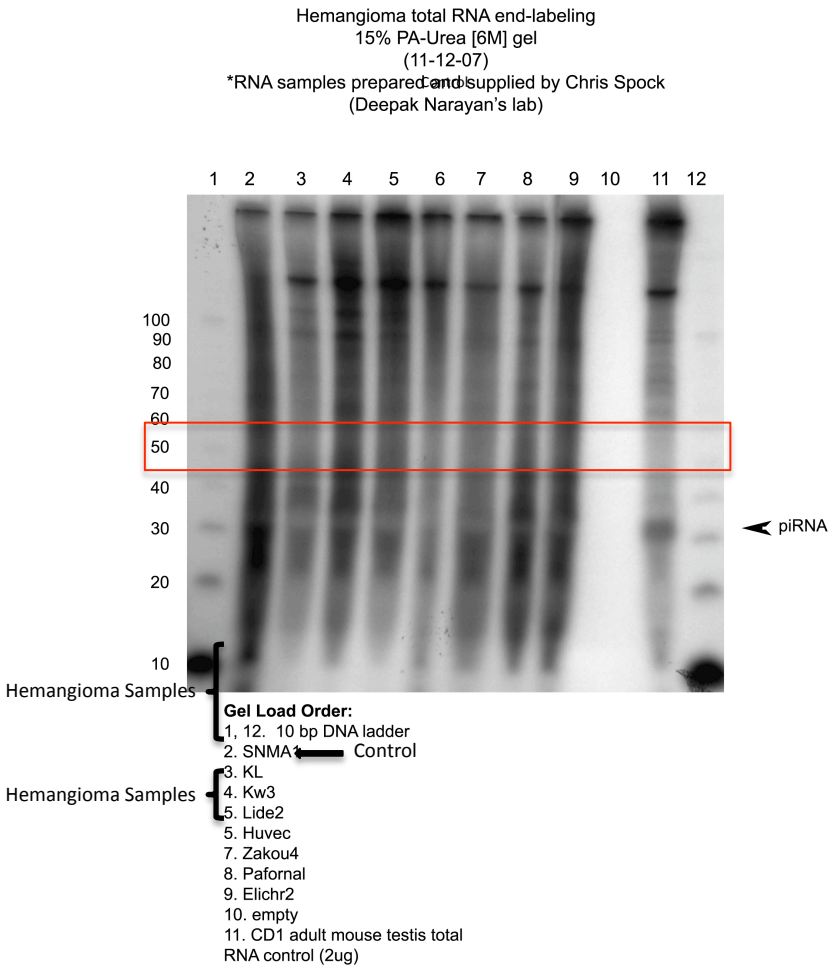
The origins of fat. A hypothetical scheme of adipocyte development is shown. C/EBPs, enhancer binding proteins.

C.R. Kahn, *Science* **322,** 543 (2008)

Appendix of Cellular Markers

Marker	Description
DLK	Also known as Pref-1, marker of pre-adipocytes, and cell surface receptor in the epidermal growth factor family
Glut-1	Glucose transporter normally expressed in the microvascular endothelium of blood-tissue barriers such as brain, retina, placenta, and endoneurium
NG2	Neural/glial cell 2, also known as chondroitin sulfate proteoglycan, is a proteoglycan associated with pericytes during vascular morphogenesis; a marker of pericytes
PDGFR	Platelet derived growth factor receptor, a marker of pericytes
PPAR-gamma	Expressed by pericytes and adipocytes
sFlt-1	Soluble form of the VEGF transmembrane receptor
SH2	Also known as CD105, a surface marker of hemangioma derived MSCs along with SH3, SH4, CD90, CD29, CD133, smooth muscle actin
CD14	Hematopoietic marker
CD31	Hematopoietic/endothelial marker
CD34	Marker of endothelial progenitor cells along with CD133, also found on MSCs; downregulated in culture
CD45	Hematopoietic marker
CD133	Also known as AC133, a marker of endothelial progenitor cells along with CD34
CD146	Also known as S-endo1, Mel-CAM, Muc18, or gicerin, an endothelial marker found at the surface of pericytes

PiRNA Expression in Hemangiomas



Expression of Factors RB and DLK as Determined by Quantitative RT-PCR

RB Expression

Sample	Age in Days	RE	+/-
Adult Endothelial Control	0	1.00	0.02
Neonatal Endothelial Control	0	0.99	0.02
Human Umbilical Vein Endothelial Control	0	1.19	0.07
Human Dermal Endothlelial Control	0	1.4	0.29
Proliferating Hemangioma	81	2.05	0.04
Proliferating Hemangioma	95	6.54	0.27
Proliferating Hemangioma	95	3.61	0.32
Proliferating Hemangioma	165	1.91	0.06
Plateau Hemangioma	286	2.63	0.02
Plateau Hemangioma	299	2.87	0.33
Plateau Hemangioma	380	1.93	0.07
Plateau Hemangioma	418	7.06	0.33
Plateau Hemangioma	420	6.39	0.22
Plateau Hemangioma	547	6.36	0.41
Plateau Hemangioma	590	1.81	0.20
Plateau Hemangioma	635	6.7	0.18
Plateau Hemangioma	752	1.85	0.11
Plateau Hemangioma	760	7.33	0.65
Involuting Hemangioma	1171	1.83	0.46
Involuting Hemangioma	1277	1.34	0.06
Involuting Hemangioma	1520	4.02	0.57
Involuting Hemangioma	2138	0.5	0.02
Involuting Hemangioma	3626	4.18	0.34

DLK Expression

Sample	Age in Days	RE	+/-
Adult Endothelial Control	0	1.00	0.03
Neonatal Endothelial Control	0	0.52	0.03
Human Umbilical Vein Endothelial Control	0	0.00	0.00
Human Dermal Endothlelial Control	0	0.00	0.00
Proliferating Hemangioma	81	308974.72	11193.45
Proliferating Hemangioma	95	70988.3	3396.23
Proliferating Hemangioma	95	153060.6	10311.93
Proliferating Hemangioma	165	596991.60	24691.21
Plateau Hemangioma	286	369600.56	14907.02
Plateau Hemangioma	299	1119257.50	153168.75
Plateau Hemangioma	380	214070.92	8838.76
Plateau Hemangioma	418	69511.45	2480.54
Plateau Hemangioma	420	81457.12	2784.75
Plateau Hemangioma	547	64348.36	2082.9
Plateau Hemangioma	590	164881.06	22057.23
Plateau Hemangioma	635	126015.95	7204.07
Plateau Hemangioma	752	567857.56	35369.21
Plateau Hemangioma	760	43438.04	1599.01
Involuting Hemangioma	1171	716259.50	176958.58
Involuting Hemangioma	1277	94.47	16.14
Involuting Hemangioma	1520	31124.69	1053.16
Involuting Hemangioma	2138	2330.59	165.33
Involuting Hemangioma	3626	560208.30	56309.02

Expression of Factors Related to Stem Cell Pluripotency as Determined by Quantitative RT-PCR

Sample	Age in Days	Oct4		Sox2		Nanog		C-Myc	
		RE	+/-	RE	+/-	RE	+/-	RE	+/-
Adult Endothelial Control	0	1.00	0.04	1.00	0.10	1.00	0.12	1.00	0.06
Neonatal Endothelial Control	0	0.41	0.04	0.00	0.00	0.78	0.03	1.88	0.04
Human Umbilical Vein Endothelial Control	0	14.72	1.64	195.26	7.79	29.4	2.1	0.00	0.00
Human Dermal Endothlelial Control	0	2.45	1.06	0.00	0.00	0.00	0.00	0.00	0.00
Proliferating Hemangioma	81	19.45	1.08	25.24	5.99	94.17	4.17	6.49	0.16
Proliferating Hemangioma	95	73.16	8.08	575.63	85.44	381.83	44.39	1.00	0.02
Proliferating Hemangioma	95	91.97	8.73	396.01	133.29	237.28	18.88	0.00	0.00
Proliferating Hemangioma	165	9.43	0.46	11.22	1.28	88.24	4.82	0.00	0.73
Plateau Hemangioma	286	15.96	0.57	74.31	9.72	282.30	6.85	12.23	0.12
Plateau Hemangioma	299	19.18	2.55	20.96	2.56	176.75	21.05	8.76	1.08
Plateau Hemangioma	380	4.34	0.09	295.36	12.48	45.27	3.95	5.76	0.07
Plateau Hemangioma	418	90.6	14.54	249.72	48.03	264.62	11.99	1.17	0.09
Plateau Hemangioma	420	27.17	2.04	0.00	0.00	658.75	42.09	1.88	0.03
Plateau Hemangioma	547	0.00	0.00	1787.73	247.59	336.11	36.82	0.78	0.04
Plateau Hemangioma	590	78.65	4.27	0.00	0.00	2012.48	156.71	35.65	3.07
Plateau Hemangioma	635	337.05	51.12	882.22	28.58	3072.5	165.83	0.00	0.00
Plateau Hemangioma	752	18.06	1.10	78.30	9.29	127.31	7.69	4.13	0.25
Plateau Hemangioma	760	107.94	16.56	442.11	108.44	697.57	39.62	1.77	0.08
Involuting Hemangioma	1171	32.69	8.19	104.41	27.44	392.09	95.60	12.92	3.21
Involuting Hemangioma	1277	8.61	0.96	511.79	63.8	14.96	0.74	0.36	0.01
Involuting Hemangioma	1520	47.69	5.91	327.01	88.11	453.72	8.66	0.62	0.03
Involuting Hemangioma	2138	6.53	0.58	963.52	137.96	33.11	0.95	0.03	0.00
Involuting Hemangioma	3626	28.60	1.11	317.89	30.83	339.93	35.40	5.43	0.48

Expression of Factors Related to IGF-2 Gene Locus as Determined by Quantitative RT-PCR

Sample	Age in Days	IGF-2		CTCF		H19		BORIS	
		RE	+/-	RE	+/-	RE	+/-	RE	+/-
Adult Endothelial Control	0	1.00	0.05	1.00	0.04	0.00	0.00	0.00	0.00
Neonatal Endothelial Control	0	0.42	0.01	0.86	0.03	0.00	0.00	0.00	0.00
Human Umbilical Vein Endothelial Control	0	16.46	2.02	3.32	0.17	0.00	0.00	0.00	0.00
Human Dermal Endothlelial Control	0	0.16	0.05	2.49	0.22	0.00	0.00	0.00	0.00
Proliferating Hemangioma	81	11175.69	244.15	2.94	0.06	1.35	0.09	0.07	0.00
Proliferating Hemangioma	95	5340.17	513.55	9.64	0.18	0.00	0.00	0.00	0.00
Proliferating Hemangioma	95	6310.24	144.79	10.57	0.41	0.00	0.00	0.00	0.00
Proliferating Hemangioma	165	7008.52	197.56	2.81	0.08	0.40	0.02	0.40	0.01
Plateau Hemangioma	286	7104.24	179.33	3.99	0.05	0.29	0.05	1.01	0.07
Plateau Hemangioma	299	9342.13	1080.98	7.41	0.93	1.00	0.23	1.00	0.17
Plateau Hemangioma	380	3725.51	151.69	2.83	0.08	0.17	0.03	0.75	0.13
Plateau Hemangioma	418	8038.53	228.03	31.7	1.59	0.00	0.00	0.00	0.00
Plateau Hemangioma	420	12583.25	209.86	13.2	0.51	0.00	0.00	0.00	0.00
Plateau Hemangioma	547	6505.15	215.46	0.00	0.00	0.00	0.00	0.00	0.00
Plateau Hemangioma	590	32721.81	2518.83	6.30	0.41	0.25	0.00	0.00	0.00
Plateau Hemangioma	635	11762.76	972.26	16.68	0.71	0.00	0.00	0.79	0.09
Plateau Hemangioma	752	10283.14	585.44	3.42	0.29	0.18	0.01	0.77	0.20
Plateau Hemangioma	760	11517.89	341.06	15.92	1.09	0.00	0.00	0.5	0.05
Involuting Hemangioma	1171	13263.79	3242.72	8.51	2.08	0.86	0.23	0.77	0.20
Involuting Hemangioma	1277	29.93	1.49	2.73	0.13	0.00	0.00	0.00	0.00
Involuting Hemangioma	1520	1162.69	35.52	7.46	0.52	0.00	0.00	0.00	0.00
Involuting Hemangioma	2138	66.49	3.2	1.48	0.06	0.00	0.00	0.00	0.00
Involuting Hemangioma	3626	21173.78	2370.70	15.67	0.90	0.13	0.02	2.34	0.57

Mean Quantitative RT-PCR Expression Levels

Group Statistics

	sample or control	N	Mean	Std. Deviation	Std. Error Mean
RB Expression	sample	19	3.7321	2.27742	.52248
	control	4	1.1450	.19330	.09665
p16 Expression	sample	19	3.2800	1.76556	.40505
	control	4	2.4925	2.43641	1.21820
DLK Expression	sample	19	276866.9100	3.04586E5	69876.81214
	control	4	.3800	.48056	.24028
Oct4 Expression	sample	19	53.5305	76.54509	17.56065
	control	4	4.6450	6.77114	3.38557
Sox2 Expression	sample	19	371.7595	447.44164	102.65015
	control	4	49.0650	97.46447	48.73224
Nanog Expression	sample	19	510.9995	762.19721	174.86003
	control	4	7.7950	14.40972	7.20486
C-myc Expression	sample	19	5.2095	8.43447	1.93500
	control	4	.7200	.90569	.45284
Igf2 Expression	sample	19	9427.1426	7615.04047	1747.01010
	control	4	4.5100	7.97440	3.98720
CTCF Expression	sample	19	8.5926	7.61084	1.74605
	control	4	1.9175	1.19092	.59546

Statistical Analysis of Quantitative RT-PCR Expression Levels

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
RB Expression	Equal variances assumed	11.750	.003	2.229	21	.037	2.58711	1.16061	.17348	5.00073
	Equal variances not assumed			4.869	19.119	.000	2.58711	.53134	1.47546	3.69875
p16 Expression	Equal variances assumed	1.272	.272	.763	21	.454	.78750	1.03210	-1.35887	2.93387
	Equal variances not assumed			.613	3.692	.575	.78750	1.28378	-2.89703	4.47203
DLK Expression	Equal variances assumed	7.333	.013	1.785	21	.089	2.76867E5	1.55129E5	-45742.34547	5.99475E5
	Equal variances not assumed			3.962	18.000	.001	2.76867E5	69876.81215	1.30061E5	4.23672E5
Oct4 Expression	Equal variances assumed	2.112	.161	1.253	21	.224	48.88553	39.01073	-32.24172	130.01278
	Equal variances not assumed			2.733	19.204	.013	48.88553	17.88403	11.48069	86.29036
Sox2 Expression	Equal variances assumed	2.223	.151	1.410	21	.173	322.69447	228.78659	-153.09330	798.48224
	Equal variances not assumed			2.840	20.715	.010	322.69447	113.63047	86.18862	559.20033
Nanog Expression	Equal variances assumed	2.221	.151	1.296	21	.209	503.20447	388.20758	-304.11739	1310.52634
	Equal variances not assumed			2.875	18.061	.010	503.20447	175.00840	135.61425	870.79470
C-myc Expression	Equal variances assumed	2.010	.171	1.044	21	.308	4.48947	4.29990	-4.45266	13.43161
	Equal variances not assumed			2.259	19.672	.035	4.48947	1.98728	.33963	8.63931
Igf2 Expression	Equal variances assumed	3.439	.078	2.429	21	.024	9422.63263	3878.43022	1356.99545	17488.26982
	Equal variances not assumed			5.394	18.000	.000	9422.63263	1747.01465	5752.29380	13092.97147
CTCF Expression	Equal variances assumed	3.312	.083	1.719	21	.100	6.67513	3.88419	-1.40249	14.75275
	Equal variances not assumed			3.618	20.747	.002	6.67513	1.84479	2.83583	10.51444

MicroRNAs Upregulated or Downregulated Compared to Controls

miRNA	Expression Compared to Controls
hsa-miR-320c	Decreased
hsa-miR-299-5p	Decreased
hsa-miR-320a	Decreased
hsa-miR-106a	Decreased
hsa-miR-126	Increased
hsa-miR-517a	Increased
hsa-miR-517b	Increased
hsa-miR-195	Increased
hsa-miR-152	Increased
hsa-miR-1308	Increased
hsa-miR-143	Increased
hsa-miR-151-3p	Increased
hsa-miR-17	Increased
hsa-miR-26b	Increased
hsa-let-7g	Increased
hsa-miR-199a-3p	Increased
hsa-miR-145	Increased
hsa-miR-1323	Increased
hsa-miR-145	Increased
hsa-let-7c	Increased
hsa-miR-21	Increased
hsa-miR-27a	Increased
hsa-miR-1238	Increased
hsa-miR-516b	Increased
hsa-miR-424	Increased
hsa-miR-522	Increased
hsa-miR-23b	Increased
hsa-miR-30e	Increased

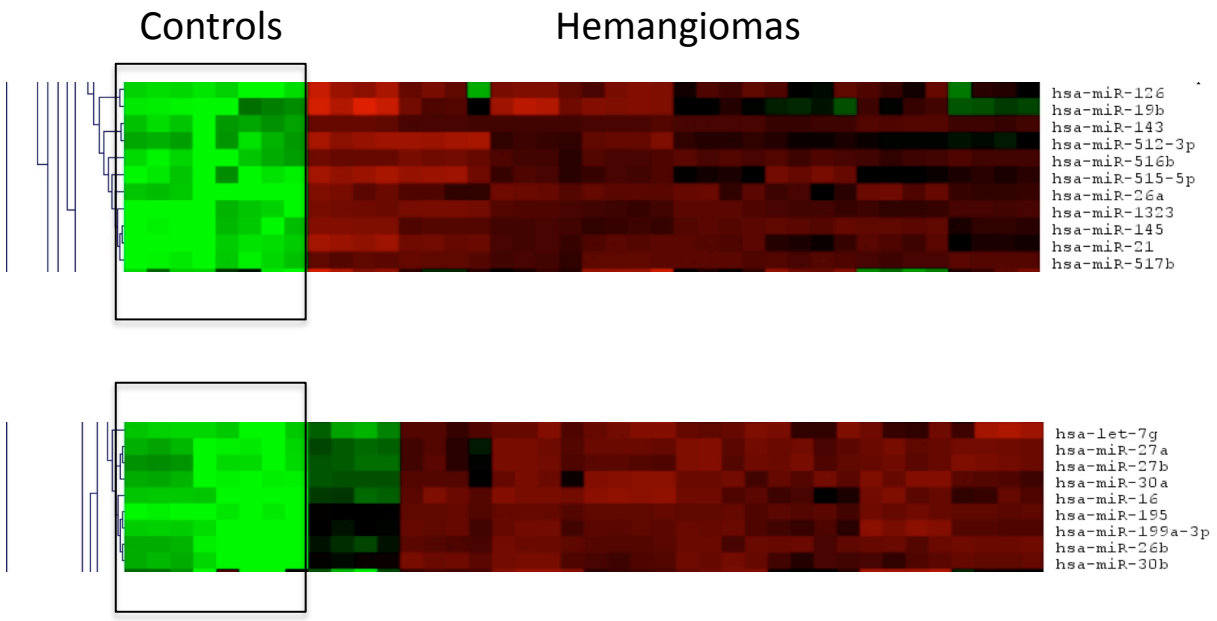
P-value < 0.000056 for all miRNAs listed

MicroRNAs Differentially Expressed in Hemangiomas

	S01 – 299 days	S02 - 1171	S03 - 752	S04 - 380	S05 - 81	S06 - 3626	S07 - 590	S08 - 286	S09 - 165	S10 - Adult	S11 - Neonatal
miRNA	Averaged Signal	Averaged Signal	Averaged Signal	Averaged Signal	Averaged Signal	Averaged Signal	Averaged Signal	Averaged Signal	Averaged Signal	Averaged Signal	Averaged Signal
hsa-miR-320c	2,523	2,099	2,227	2,051	5,116	1,813	2,091	2,444	2,168	8,777	9,007
hsa-miR-299-5p	71	143	72	100	148	70	119	111	150	323	319
hsa-miR-320a	2,729	2,261	2,343	2,073	5,792	1,884	2,258	2,368	2,174	9,048	9,384
hsa-miR-106a	1,466	771	920	1,078	876	1,259	661	1,505	1,244	2,430	2,392
hsa-miR-126	33,777	27,775	31,183	31,147	37,147	34,761	30,094	33,915	30,145	18,571	17,781
hsa-miR-517a	15,488	14,731	11,029	14,639	13,220	7,615	12,725	11,341	15,407	106	229
hsa-miR-517b	13,095	11,903	9,829	12,034	12,860	6,421	11,051	9,261	13,233	132	250
hsa-miR-195	17,463	16,937	18,984	16,854	8,154	19,179	15,630	17,371	16,912	529	605
hsa-miR-152	1,734	1,140	1,756	1,508	1,650	2,025	1,352	1,529	1,334	433	401
hsa-miR-1308	5,567	3,043	2,355	682	2,504	461	7,489	297	1,689	14,134	13,322
hsa-miR-143	16,484	12,837	18,717	15,233	25,105	16,051	12,195	13,961	14,998	51	230
hsa-miR-151-3p	780	361	682	408	1,117	434	467	472	471	1,856	1,758
hsa-miR-17	1,603	835	1,039	1,213	935	1,313	650	1,645	1,303	2,689	2,825
hsa-miR-26b	19,662	23,339	21,834	21,485	8,862	25,726	21,033	22,603	19,095	2,646	1,012
hsa-let-7g	17,303	18,409	15,822	16,696	9,198	17,300	16,073	16,611	15,576	7,468	6,997
hsa-miR-199a-3p	13,729	13,044	17,100	14,238	8,640	13,540	11,911	14,504	13,825	2,637	1,650
hsa-miR-145	12,871	10,978	20,338	18,923	33,129	21,896	20,220	14,934	17,926	130	398
hsa-miR-1323	7,471	8,094	6,790	9,722	13,136	4,980	7,742	8,853	14,050	120	344
hsa-miR-145	12,871	10,978	20,338	18,923	33,129	21,896	20,220	14,934	17,926	130	398
hsa-let-7c	41,779	34,520	31,714	33,236	29,602	32,934	36,362	41,166	33,698	19,542	23,402
hsa-miR-21	26,068	17,224	24,874	26,178	45,450	19,240	18,898	23,072	30,768	1,273	2,403
hsa-miR-27a	9,873	10,233	9,910	10,545	3,755	9,024	9,454	10,173	7,340	2,181	1,402
hsa-miR-1238	41	31	55	31	61	41	36	47	22	126	133
hsa-miR-516b	3,801	3,602	3,265	4,021	5,226	1,113	3,746	3,322	5,797	66	184
hsa-miR-424	6,514	4,575	4,459	5,923	3,174	2,917	3,849	7,070	8,222	62	119
hsa-miR-522	2,192	2,660	1,721	2,380	2,220	1,282	2,366	2,034	3,333	81	317
hsa-miR-23b	17,195	20,103	19,544	22,609	18,947	25,363	23,866	21,553	21,923	14,634	14,320
hsa-miR-30e	1,500	1,283	1,238	1,382	320	1,590	1,427	1,842	1,200	211	219

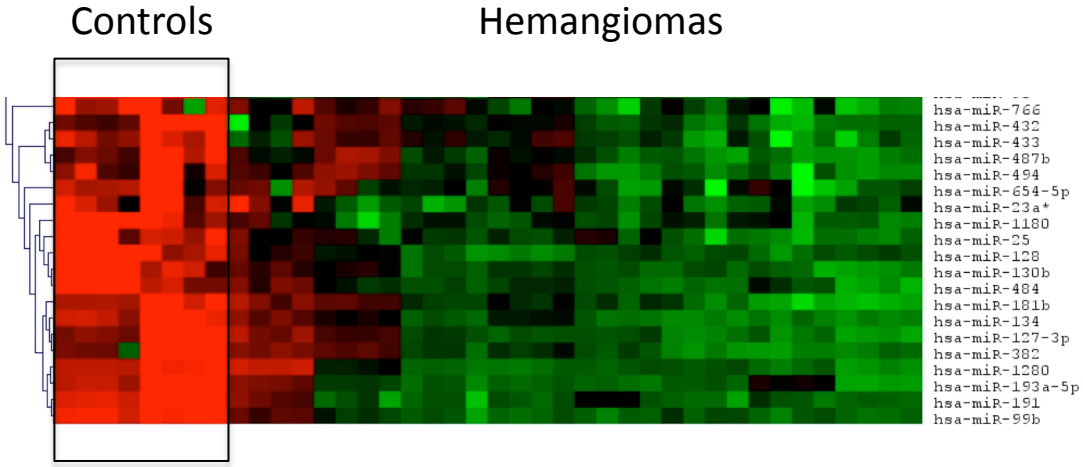
microRNA Cluster Analysis

Upregulated miRNAs in Hemangiomas



microRNA Cluster Analysis

Downregulated miRNAs in Hemangiomas



Biological Functions Implicated in Hemangioma Pathogenesis as Identified by Ingenuity Pathway Analysis Software

Top Bio Functions

Diseases and Disorders

Name	p-value	# Molecules
Cancer	1.67E-28 - 2.44E-02	55
Reproductive System Disease	1.67E-28 - 2.44E-02	45
Genetic Disorder	4.60E-23 – 9.79E-03	22
Skeletal and Muscular Disorders	4.60E-23 – 1.26E-02	26
Inflammatory Disease	1.14E-21 – 9.47E-03	20

Molecular and Cellular Functions

Name	p-value	#Molecules
Cellular Growth and Proliferation	4.21E-15 – 1.35E-02	31
Cellular movement	3.16E-06 – 1.38E-02	17
Celular Assembly and Organization	6.19E-06 – 9.79E-06	10
Cell-To-Cell Signalling and Interaction	1.59E-05 – 9.79E-03	17
Cellular Development	2.37E-05 – 1.30E-02	17

Physiological System Development and Function

Name	p-value	# Molecules
Cardiovascular System Development and Function	1.86E-07 - 1.16E-02	13
Organismal Development	2.22E-06 - 9.46E-03	8
Nervous System Development and Function	6.19E-06 - 9.79E-03	9
Tumor Morphology	1.25E-05 - 1.13E-02	11
Endocrine System Development and Function	2.37E-05 - 9.79E-03	4

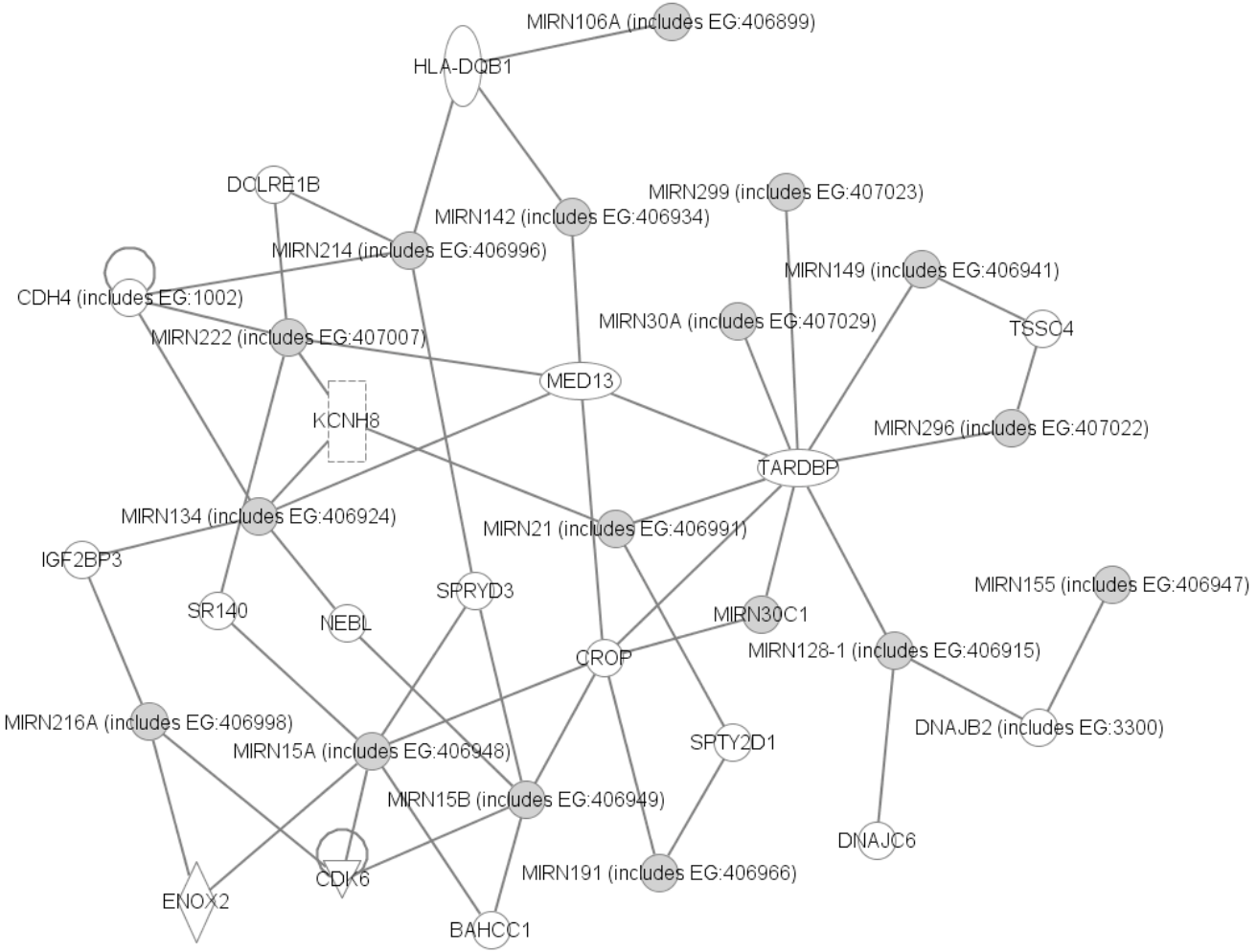
Biological Networks Implicated in Hemangioma Pathogenesis as Identified by Ingenuity Pathway Analysis Software

- **Top Networks**

ID	Associated Network Functions	Score
1	Cancer, Reproductive System Disease, Cellular Growth and Proliferation	34
2	Genetic Disorder, Skeletal and Muscular Disorders, Inflammatory Disease	31
3	Cardiovascular System Development and Function, Organism Development, Cellular Movement	29
4	Cancer, Cell Death, Skeletal and Muscular Disorders	17
5	Cellular Assembly and Organization, Nervous System Development and Function, Cellular Movement	16
6	Genetic Disorder, Skeletal and Muscular Disorders, RNA Post-Transcriptional Modification	9

Score = -log of the p-value

Network 1: Cancer, Reproductive System Disease, Cellular Growth and Proliferation – Generated by Ingenuity Pathway Analysis

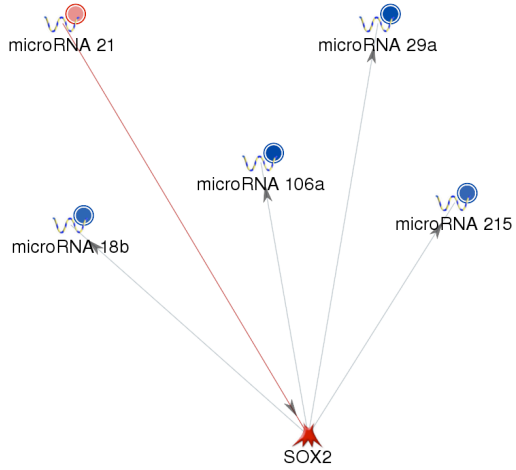


Lines indicate miRNA binds to mRNA of indicated gene.

17 focus molecules in network with 34 nodes. Top functions are
Cancer, Reproductive System Disease, Cellular Growth and Proliferation

Regulation of C-Myc

Regulation of p53 and Sox2



Hemangioma-related Gene Analysis

The following genes were either reported in the MetaCore disease database as associated with hemangiomas or were found in the literature.

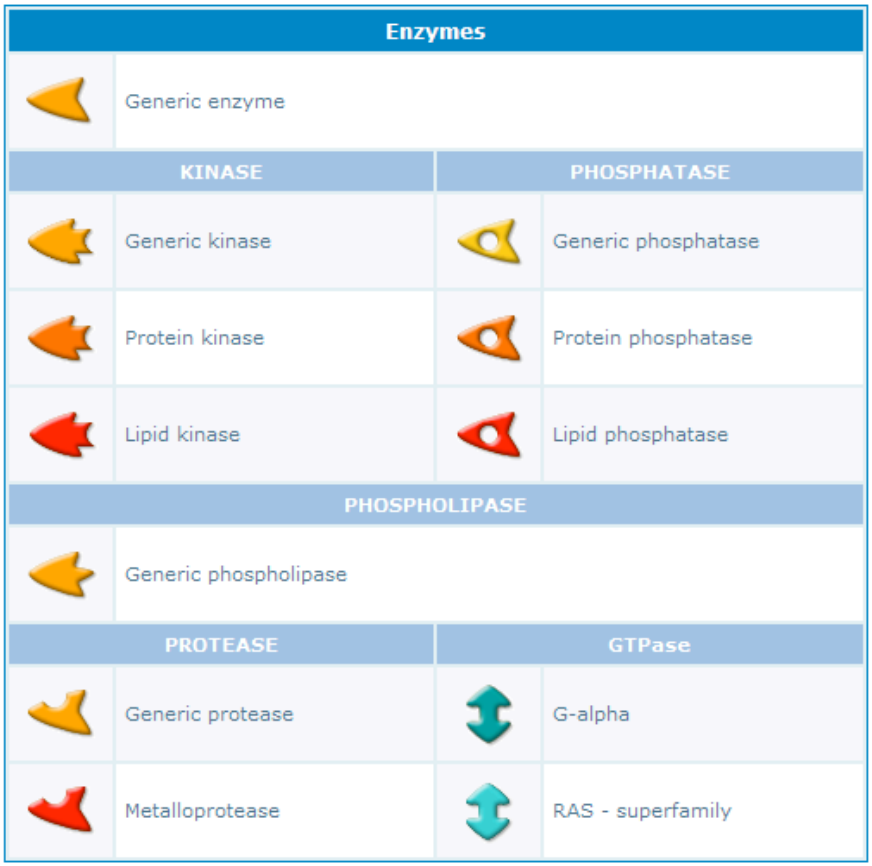
They were added to the miRNA gene set to strengthen the network analysis.

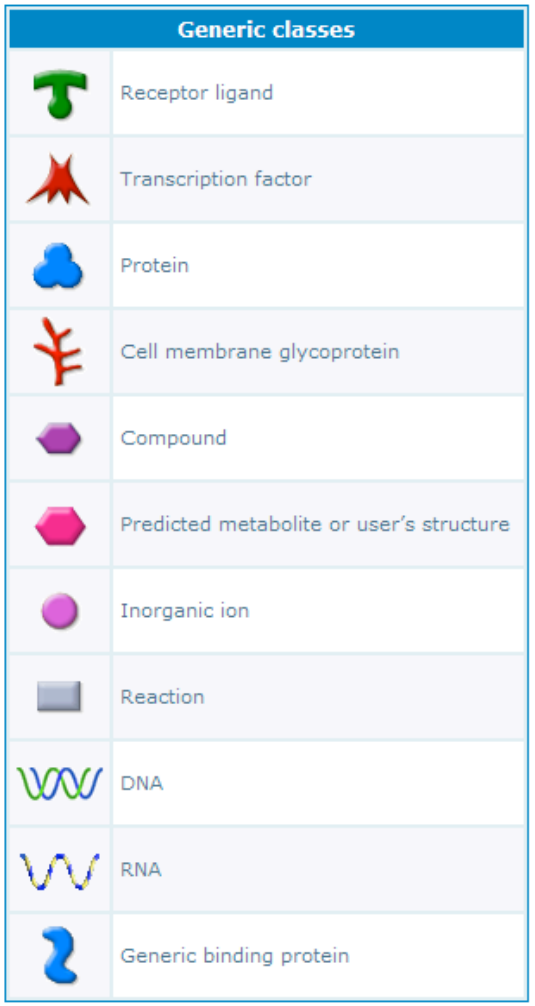
Capillary hemangioma related genes
ATR/TEM8
Angiopoietin 2
Aquaporin 1
BIC
BORIS
CTCF
CXCR4
Clusterin
IGF-2
ITGA5
ITGAV
KIP2
LYVE-1
NF-AT1(NFATC2)
NF-AT2(NFATC1)
NF-AT3(NFATC4)
NF-AT4(NFATC3)
NF-AT5
Neprilysin
TIE
TIE2
VEGFR-1
VEGFR-2
VHL
Zac1

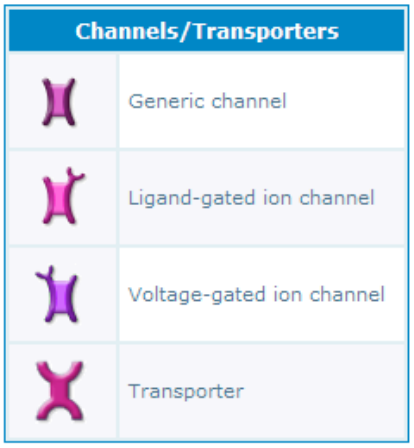
Interactome Analysis

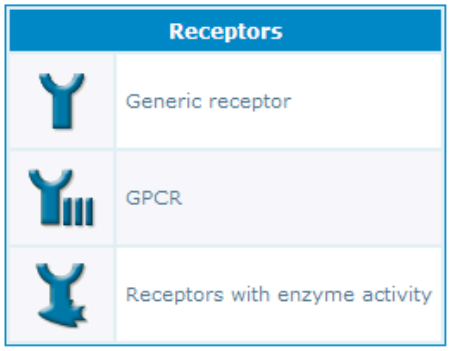
Object name	r	n	R	N	mean	z-score	p-value
SP1	11	25	1283	19665	1.63107	7.592231	1.66E-07
SP3	6	25	333	19665	0.423341	8.649733	3.04E-06
ETS1	5	25	229	19665	0.291126	8.783852	9.01E-06
ELF2	2	25	4	19665	0.005085	27.99501	9.3E-06
CTCF	3	25	34	19665	0.043224	14.24285	1.06E-05
SP4	2	25	28	19665	0.035596	10.42565	0.000575
SIL	1	25	1	19665	0.001271	28.02856	0.001271
ZBTB38	1	25	1	19665	0.001271	28.02856	0.001271
EGR1	3	25	172	19665	0.218663	5.977771	0.001313
C/EBPalpha	3	25	185	19665	0.235189	5.731578	0.001618
SRF	3	25	209	19665	0.2657	5.336239	0.00229
ETS2	2	25	57	19665	0.072464	7.175259	0.002373
EPAS1	2	25	58	19665	0.073735	7.108625	0.002455
ZBTB4	1	25	2	19665	0.002543	19.79446	0.002541
Sin3A	1	25	2	19665	0.002543	19.79446	0.002541
HOXB5	1	25	2	19665	0.002543	19.79446	0.002541
AP-2A	3	25	226	19665	0.287312	5.093282	0.002858
CTIP2	1	25	3	19665	0.003814	16.14192	0.003809
HOXD3	1	25	3	19665	0.003814	16.14192	0.003809
PLAG1	1	25	4	19665	0.005085	13.96183	0.005076
LRRFIP1	1	25	4	19665	0.005085	13.96183	0.005076
HEY1	1	25	5	19665	0.006356	12.4722	0.006341
FOXC2	1	25	5	19665	0.006356	12.4722	0.006341
ELF4	1	25	7	19665	0.008899	10.51449	0.008867
HOXA9	1	25	7	19665	0.008899	10.51449	0.008867
Oct-6	1	25	8	19665	0.01017	9.823042	0.010127
c-Fos	2	25	121	19665	0.153827	4.724569	0.01027
HOXA13	1	25	9	19665	0.011442	9.249593	0.011386
HIF1A	2	25	132	19665	0.167811	4.490431	0.012126

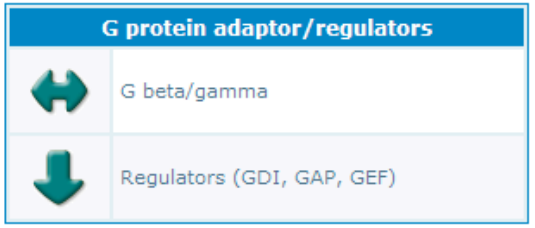
Object name = the network object name; **r =** number of network objects derived from genes from active experiment(s) that have interactions with the given network object; **n** number of network objects derived from genes in active experiment(s); **R =** total number of gene-based network objects in the complete database that have interactions with the given network object; **N =** total number of gene-based network objects in the complete database; **mean** = mean value for hypergeometric distribution (n*R/N); **z-score** = z-score ((r-mean)/sqrt(variance)); **p-value** = probability to have the given value of r or higher





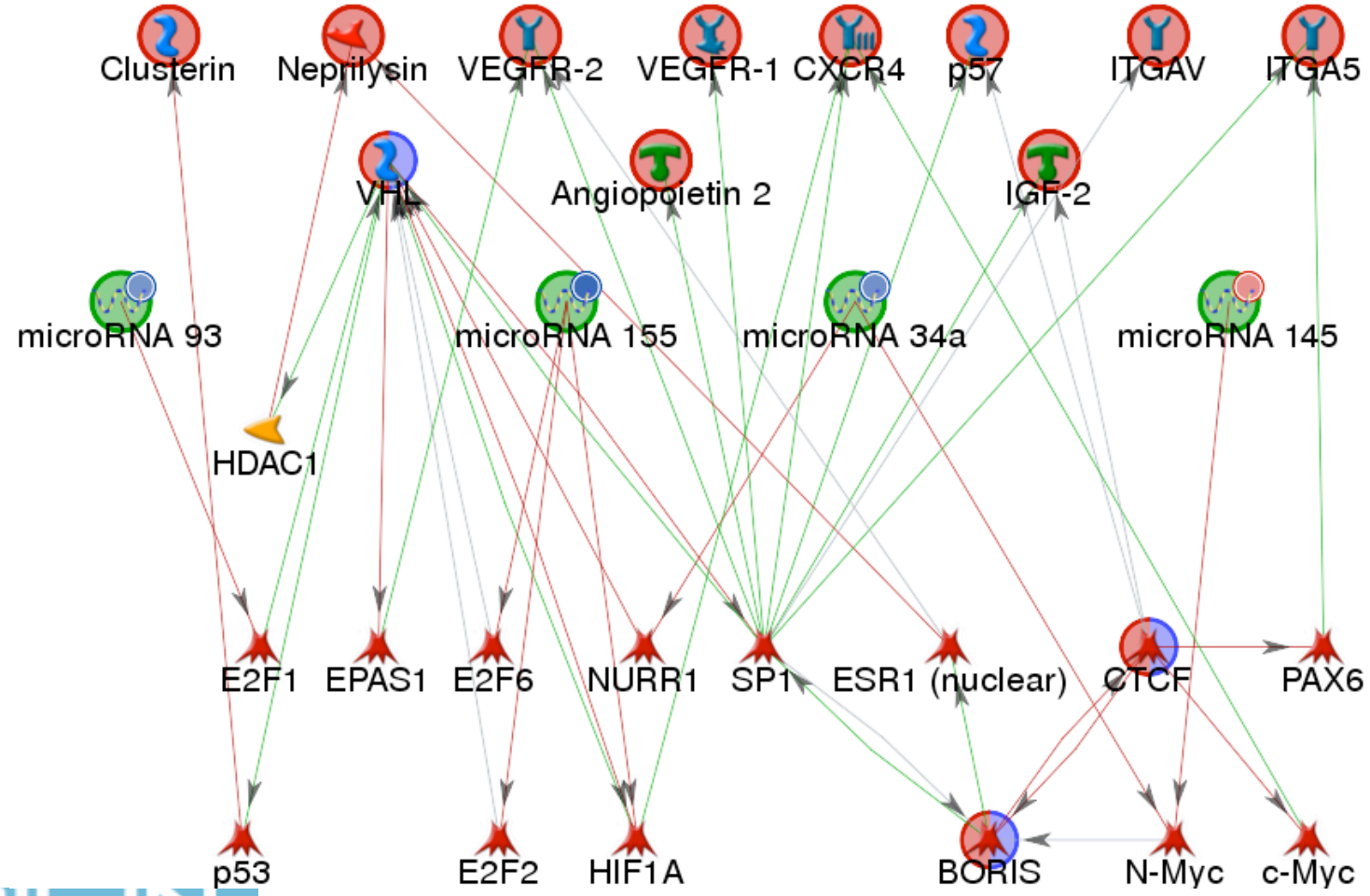




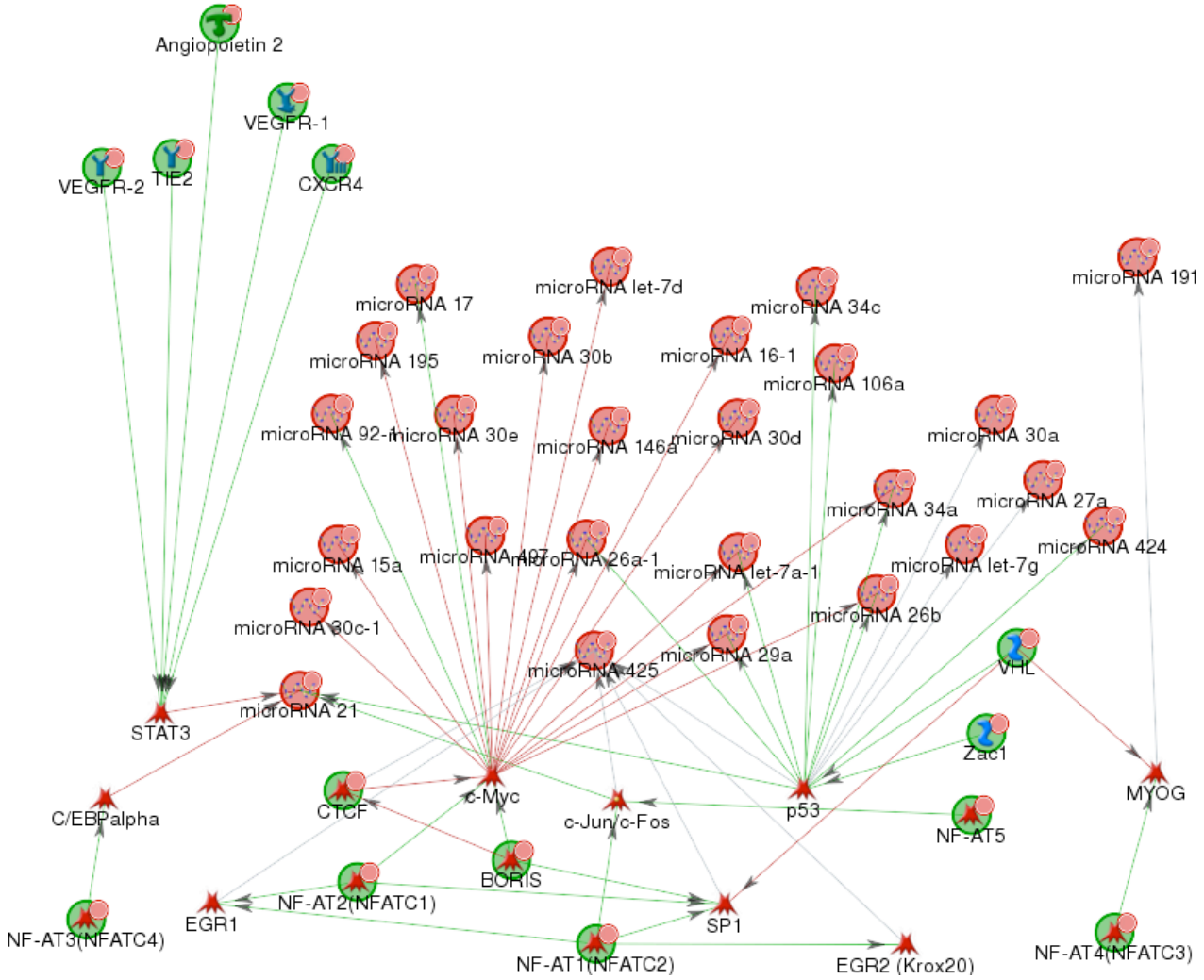


This is the key for the icons in the figures that follow.

Known hemangioma genes restricted to be endpoints (inside red circles), miRNA genes forced to be start points (inside green circles); using shortest pathway algorithm. miRNA list restricted to 4x threshold, for simplicity



Reverse of previous analysis: Shortest path from hemangioma related proteins to miRNA proteins (1.5x threshold)



Diseases Ranked According to Biomarkers (only miRNA list)

Disease	ratio		p-value
Uterine Neoplasms	13	541	9.73E-07
Uterine Diseases	13	545	1.06E-06
Leukemia, B-Cell, Chronic	3	24	2.04E-04
Neoplasms by Site	30	3865	4.45E-04
Genital Neoplasms, Female	13	981	5.43E-04
Head and Neck Neoplasms	10	618	6.07E-04
Neuroblastoma	8	404	6.35E-04
Neuroectodermal Tumors, Primitive, Peripheral	8	415	7.58E-04
Thyroid Neoplasms	6	245	1.11E-03
Adnexal Diseases	13	1072	1.27E-03
Neuroectodermal Tumors, Primitive	8	486	2.10E-03
Neoplasms, Neuroepithelial	12	992	2.11E-03
Leukemia, B-Cell	3	65	3.82E-03
Papillary Thyroid Carcinoma	3	69	4.53E-03
Eczema	2	24	5.99E-03

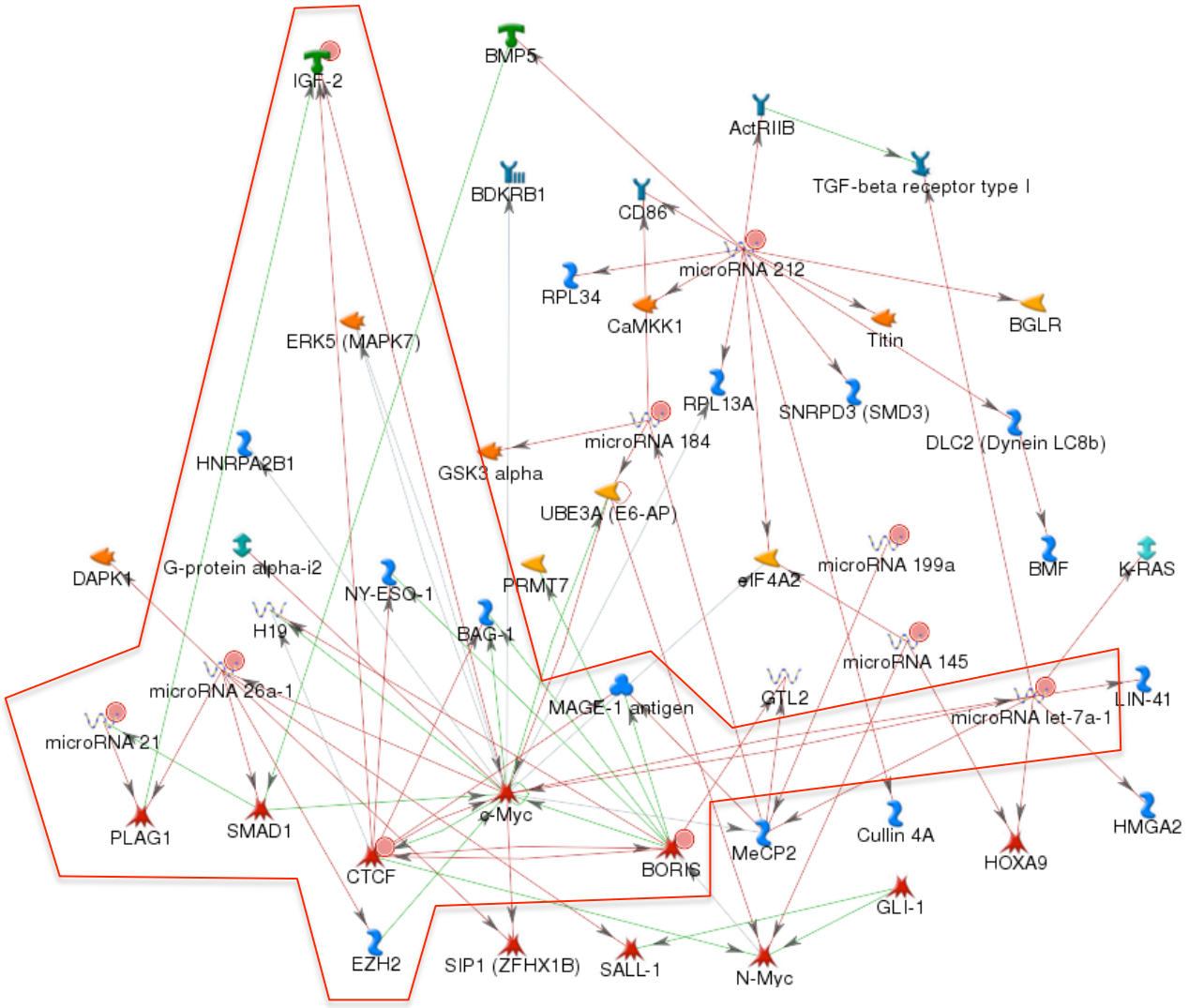
Ratio represents number of biomarkers found in sample compared to known biomarkers contained in the database for the given disease

The p-value given is for picking a random network of the same size from the database having the same or greater number of genes from the target list.

Diseases Ranked According to Biomarkers (miRNA list + Hemangioma Related Proteins)

Disease	ratio		p-value
Hemangioma	14	30	2.17E-23
Hemangioma, Capillary	9	9	2.44E-20
Hemangioblastoma	8	8	3.95E-18
Uterine Diseases	24	545	6.85E-14
Neoplasms, Vascular Tissue	14	183	2.20E-11
Uterine Neoplasms	21	541	4.53E-11
Genital Neoplasms, Female	26	981	5.15E-10
Adnexal Diseases	26	1072	3.57E-09
Endocrine Gland Neoplasms	27	1245	1.78E-08
Head and Neck Neoplasms	19	618	2.38E-08
Neoplasms by Site	48	3865	6.61E-08
Thyroid Neoplasms	12	245	1.09E-07
Digestive System Neoplasms	33	1996	1.59E-07
Thyroid Diseases	14	372	2.19E-07
Nervous System Neoplasms	13	326	3.30E-07

Densest Pathway Network for Proteins Common with Uterine Disease as Identified by MetaCore



The network centered around miRNAs let-7a1, 26a-1, 21 and the transcription factors BORIS, and CTCF

P = 3.13e-22

The p-value given is for picking a random network of the same size from the database having the same or greater number of genes from the target list