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Research Article

Mesenchymal Stromal Cells and Vascular Morphogenesis: Gene Expression Profiles and Promoting Pathways

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ABSTRACT

Objective: Hematopoietic cells and mesenchymal stromal cells are closely related to endothelial cells in the embryological cell differentiation lineages. To study the pathobiology of vascular immunology and microenvironment in vascular morphogenesis, we analyzed the genetic factors known to be involved in vascular anomalies in humans and mice in the expression data from the Immunological Genome Project (ImmGen).

Methods: We mined the Pictures of Standard Syndromes of Undiagnosed Malformations and NCBI Online Mendelian Inheritance in Man databases to construct a gene list related to vasculature. We studied the expression signatures of these genes in the ImmGen database. Hierarchical clustering analyses were performed using Partek® Genomics Suite 6.6. Next, the acquired clusters were separately investigated within Ingenuity Pathway Analysis (IPA). Based on these results we performed a Principal Component Analysis (PCA) with pericyte samples from a separate database to investigate the relation with pericytes.

Results: Our database queries resulted in a gene list of 438 genes related to vasculature, of which 384 could be studied within the ImmGen data set. Through hierarchical clustering we identified five distinct clusters of which one was specific for expression in mesenchymal cell lines. Next, using IPA we found various pathways related to pericyte functions. A subsequent PCA with pericyte samples showed a close resemblance to specific stromal cells of mesenchymal origin indicating shared expression profiles for vascular genes between pericytes and these cell types. These results indicate that the processes of Epithelial-Mesenchymal-Transition and or Endothelial-Mesenchymal-Transition underly the interaction between epithelial/endothelial cells and mesenchymal stromal cells in vascular morphogenesis.

Conclusion: In this data analysis study, we performed data fusion from various sources that may aid future mechanistic and therapeutic studies in study design and cell type selection as well as provide a potential strategy to find therapeutic targets based on the specific pathological molecular mechanisms related to vascular anomalies.

Keywords: Vascular morphogenesis; Data mining Pericytes; Epithelial-mesenchymal-transition; Endothelial-mesenchymal-transition; Hemangioma

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INTRODUCTION

Angiogenesis and vascular morphogenesis are fundamental processes underlying the formation of new blood vessels. Angiogenesis starts with a stimulus, leading to endothelial sprouting, vascular branching and elongation, followed by lumen formation and vessel maturation which requires the recruitment of mural cells and the generation of Extra Cellular Matrix (ECM) [1-3]. This multi-step process of vessel formation is highly regulated by many different cellular mechanisms and various cell types [4,5].

Mesenchymal Stem Cells (MSC) play a particular important role in angiogenesis as they contribute to blood vessel formation and stabilization, generating a long-lasting functional vasculature in both normal and pathological circumstances. Another important cell type is the pericyte that originates from MSCs [6-9]. These cells are embedded within the vascular walls and regulate vascular development, angiogenesis and vascular permeability [10]. Pericyte dysfunction is observed in vascular diseases such as stroke but also pericyte-induced angiogenesis is an important factor in tumor development and growth [11-13].

Abnormal vascular formation can be observed in many congenital syndromes. Especially the skin is involved in defects in vascular morphogenesis [14]. Over the years, many genetic loci have been attributed to pathological vascular malformations in vascular anomalies and genetic causes are used to classify vascular malformations and tumors and are listed in the International Society for the Study of Vascular Anomalies (ISSVA) classification [15,16]. These genetic causes have helped to understand the pathogenesis and open up the path towards precision medicine and targeted therapies. Moreover, it also sheds a light towards the complexity and the cellular mechanisms involved. For example, as vascular malformations are a prominent comorbidity of various primary immunodeficiencies (i.e.PTEN hamartoma tumor syndrome and Ataxia-Telangiectasia), we know that the immune system is an important contributor in the context of (stem) cell differentiation and vessel formation [17-21].

In this study, we try to integrate the genetic factors known to be involved in vascular formation and study the expression and common pathways of these genes in detail to find potential new targets for therapeutic agents for vascular anomalies. We mined various databases to acquire the relevant genetic factors and subsequently analyzed these genetic factors in the gene expression data from the Immunological Genome Project (ImmGen) composed of hematopoietic and mesenchymal cells.

METHODS

Data sources (Phenotype to genotype)

First, we queried the POSSUM database to draw phenotypes from the category and subcategories of skin vascular changes. These acquired phenotypes from POSSUM were next searched in the NCBI Online Mendelian Inheritance in Man (OMIM) database to collect the mutated genes underlying the phenotypes [22,23]. To extend our gene set, we also searched the MGI database for genes responsible for vascular phenotypes as listed by the 2018 ISSVA classification [16,24]. An overview of the selected phenotypic sub-categories and mutations from human and mouse genes for this analysis can be found in the Supplementary data Tables 1 and 2.

Next, we wanted to investigate the expression of these genes in

Table 1: Most significant canonical pathways predicted to be involved by Ingenuity Pathway Analysis (IPA) per cluster and calculated P-value.

	Top canonical pathways	Computed P-value in IPA
Cluster All	Regulation of the epithelial-mesenchymal transition pathway	1.03E-27
	Molecular mechanisms of cancer	4.81E-27
	Human embryonic stem cell pluripotency	4.91E-26
	Hepatic fibrosis/hepatic stellate cell activation	9.38E-25
	Integrin signaling	3.68E-23
Cluster 1	Human embryonic stem cell pluripotency	1.67E-11
	Axonal guidance signaling	3.82E-08
	Role of NANOG in mammalian embryonic stem cell pluripotency	3.92E-08
	Molecular mechanisms of cancer	4.27E-08
	Regulation of the epithelial-mesenchymal transition pathway	8.53E-08
Cluster 2	NGF signaling	2.55E-13
	ERK/MAPK signaling	4.46E-13
	Integrin signaling	1.65E-12
	Hereditary breast cancer signaling	2.63E-12
	B Cell receptor signaling	3.27E-12

Cluster 3	Mouse embryonic stem cell pluripotency	1.93E-05
	Glioblastoma multiforme signaling	**9.43E-05**
	Factors promoting cardiogenesis in vertebrates	3.62E-04
	Polyamine regulation in colon cancer	5.04E-04
	Estrogen-mediated S-phase Entry	6.01E-04
Cluster 4	ILK signaling	1.93E-13
	Molecular mechanisms of cancer	7.62E-09
	PPAR/RXR Activation	1.36E-08
	Regulation of the epithelial-mesenchymal transition pathway	2.29E-08
	IL-8 signaling	3.28E-08
Cluster 5	Hepatic fibrosis/hepatic stellate cell activation	3.58E-17
	Human embryonic stem cell pluripotency	1.35E-07
	STAT3 pathway	5.59E-07
	Regulation of the epithelial-mesenchymal transition pathway	1.13E-06
	Intrinsic prothrombin activation pathway	5.89E-06

Table 2: Most significant canonical pathways as predicted by Ingenuity Pathway Analysis (IPA) and underlying genes that are specifically overexpressed in stromal cell lineages (Cluster 5) in the ImmGen database.

Top canonical pathway	Genes	Computed P-value in IPA
Hepatic fibrosis/hepatic stellate cell activation	Col15a1 Col3a1 Cyp2e1 Kdr	3.58E-17
	Col18a1 Col5a1 Fgf2 Pdgfra	
	Col1a1 Col5a2 Fgfr2 Pdgfrb	
	Col1a2 Ctgf Flt1 Serpine1	
Human embryonic stem cell Pluripotency	Bmp4 Fgfr2 Pdgfrb Tcf7l1	1.35E-07
	Fgf2 Pdgfra S1pr3 Wnt5a	
STAT3	Fgfr2 Kdr Pdgfrb Flt1	5.59E-06
	Pdgfra Tgfbr3	
Regulation of the epithelial-mesenchymal transition pathway	Fgf2 Foxc2 Pdgfrb Twist2	1.13E-06
	Fgfr2 Notch3 Tcf7l1 Wnt5a	

their biological context. We used the data from the ImmGen as this dataset contains detailed gene expression data of both mice and human, from many hematopoietic and stromal lineages [25]. The ImmGen dataset was downloaded from the Gene Expression Omnibus (GEO) database under accession number GSE15907. Another dataset was downloaded to validate our observations in the ImmGen data and further study the gene expression in pericytes (GSE71535). To assess the pathogenicity of the genes in the gene set, we looked up the constraint information of the gene in the ExAC database which is now incorporated in the Genome Aggregation Database (gnomAD) and used in our sequencing projects [26,27]. The consortium computed constraint statistics, based on exome DNA sequencing data and subsequent CNV analysis of 60,706 individuals, which indicate the probability that a gene is intolerant to a loss-of-function mutation.

Data analysis

Raw intensity values of 681 Immgen samples were normalized by RMA normalization (Robust Multichip Analysis) using Partek® Genomics Suite 6.4 (Partek Inc., St. Louis, MO). Human genes were converted into mouse gene analogues using their Entrez IDs. From this list, 437 probe sets were found on the Affymetrix array and summarized into 384 genes by taking the median of expression intensity values. Log2 expression values of 681 cell lineages with 384 genes were standardized into z-scores prior to the clustering analysis. Hierarchical clustering was performed by using Partek® Genomics Suite 6.6. This method uses an agglomerative algorithm in which each record is treated as an individual cluster

to begin with. We used Pearson's dissimilarity (d) to calculate the distance between two samples: d=(1-r)/2 where r is the Pearson's correlation coefficient. Clusters were then merged two at a time based on Ward's method until there was a single cluster containing all records [28]. We noticed a single cluster (right-most orange cluster) with genes that were highly expressed in stromal cells, so we performed another hierarchical clustering analysis using only genes in this cluster to investigate further. A PCA was used to visualize the clustering of all the samples in the context of these 384 vascular genes, including 681 cell lineages of Immgen (GSE15907) and two adipose tissue-derived pericyte samples (GSE71535) after quantile normalization (Figure 1).

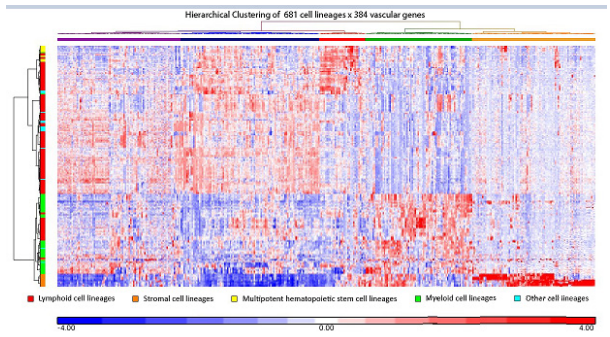

Figure 1: Hierarchical clustering of 681 Immgen cell lineages and 384 vascular genes. Column dendrogram shows the gene clusters: from left to right cluster 1 (purple), cluster 2 (blue), cluster 3 (red), cluster 4 (green) and cluster 5 (orange). Row dendrogram shows clustering of the lineages: lymphoid line (red), stromal cells (orange), multipotent hematopoietic stem cell (yellow), myeloid line (green), and other (blue). The heat map shows the spectrum of expression values as the z-score of the log2 expression values for each gene; red indicates relative overexpression and blue represents relative under expression..

Functional annotation

Functional annotation was performed using QIAGEN/Ingenuity Pathway Analysis software (QIAGEN, Redwood City, CA). Within IPA, p-values for canonical pathways, regulators and networks are predicted based on the overlap with their knowledge archive. Pathways and networks describing all gene clusters were further analyzed for clinical significance in vascular biology.

RESULT

Expression profiles of the vascular gene list

We found 139 vascular phenotypes in Pictures of Standard Syndromes of Undiagnosed Malformations (POSSUM) and subsequently 341 genes (141 unique genes) encodingtranscripts related vascular changes. During our query in the Mouse Genome Informatics (MGI) database we found 257 phenotypes and subsequently 557 genes (297 unique genes) andhere was limited overlap (3%; 13/438) between the human and mouse vascular gene lists (Supplementary Tables 1 and 2). To assess the gene expression in immune cells and stromal cells with immunological hallmark, we performed a cluster analysis. Of the 438 genes, a total of 384 vascular genes could be converted to mouse gene analogues and were available within the ImmGen dataset for gene expression investigation (Figure 1). The 384 genes were clustered into five subgroups with different gene signatures. Cluster 1 (purple in Figure 1) and cluster 2 (blue) showed overexpression in lymphoid cells and under expression in stromal cells. On the other hand, genes in cluster 3 (red) showed the highest upregulation for the hematopoietic stem and progenitor cells. Furthermore, genes in cluster 4 (green) were upregulated in stromal cells, but also shared expression in leukocytes, mainly in the myeloid cells. The last cluster (orange) was characterized by overexpression in the stromal cells exclusively, including MSCs and endothelial cells.

Functional pathway analysis of gene signatures

To assess the gene functions and pathways underlying the entire gene list and also each individual cluster, we used Ingenuity Pathway Analysis (IPA). Top canonical pathways and computed p-values by IPA for the overall analysis and each cluster separately are shown in Table 1. The most significant enrichment in all 384 vascular genes, encoded products in the pathways of Epithelial Mesenchymal Transition, Molecular Mechanisms of Cancer, Human Embryonic Stem Cell Pluripotency, Hepatic fibrosis/Hepatic Stellate Cell activation and Integrin Signaling pathway. The underlying gene names of these pathways can be found in (Supplementary Table 3). Interestingly, there was significant overlap in the enriched pathways between the individual clusters. For example, Epithelial–Mesenchymal Transition (EMT) regulation was expected to be involved in three separate clusters (cluster 1, 4 and 5). Moreover, we noticed a substantial amount of pathways related to the neuronal system. These pathways were mainly enriched in clusters 1, 2 and 3. All summarized pathways were highly significant ($P<0.05$) implying a more than expected enrichment based on the submitted gene set for these pathways.

Transcriptional signature of stromal subsets

We further investigated gene clusters 4 and 5, since genes in these clusters showed expression in stromal cells and are therefore probably directly related to vasculature. As cluster 5 showed exclusive overexpression in stromal cells whereas genes in cluster 4 were also expressed in other cell lineages, we performed further analyses on cluster 5. We performed another cluster analysis using the 88 genes in cluster 5 to look in more detail at the expression in individual stromal cell lineages (Figure 2). We focused on the stromal cells within the ImmGen dataset, which contained cell lineages of Lymphoid Endothelial Cells (LECs), Blood Endothelial Cells (BECs), Epithelial Cells, Fibroblastic Reticular Cells (FRCs), Fibroblasts and Double Negative Cells (DNs). Most of the 88 genes were down-regulated (anti-correlated) in epithelial cells.

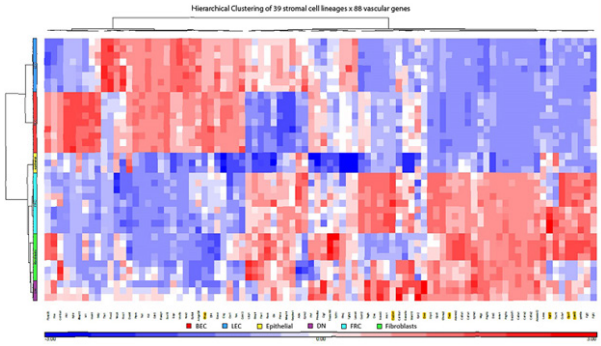

Figure 2: Hierarchical clustering of stromal cells and 88 vascular genes (Cluster 5 in Figure 1). Row dendrogram shows clustering of the genes. Genes highlighted in yellow have CNV=NaN in the ExAC database. Column dendrogram shows clustering of the stromal cell lineages: BEC: Blood Endothelial Cell (red); LEC: Lymphatic Endothelial Cell (blue); Epithelial cell (yellow); DN: Double Negative cells (purple); FRC: Fibroblastic Reticular Cell (light blue) and Fibroblasts (light green).

As we noticed multiple clusters were enhanced for EMT regulation, we specifically looked at transcription factors in our vascular gene set known in the context of EMT. The transcription factors TWIST, SOX, GATA and SNAI2, known for driving EMT, were identified in different stromal subsets. This is consistent with the negative down-regulation of epithelial cells observed in this cluster. Inducers of EMT, Notch and Wnt signaling, and BMP proteins (as part of TGF–β signaling), were likewise activated.

Transcriptional distances between pericytes and immunological cell lineages

We recognized that most of the enriched pathways found in cluster 5 resembled global functions of pericytes. Table 2 lists the enriched genes and their encoded molecules. To confirm whether the mesenchymal stromal cells in the ImmGen database resemble pericytes, we performed a principal component analysis (PCA) analysis of the 681 cell lineages combined with two pericyte samples from another dataset based on the 384 vascular genes (Figure 3). PCA analysis further highlighted the distinction of stromal cells from the hematopoietic cell populations profiled as expected. Interestingly, the two pericyte samples were grouped closely with stromal cells and were very distinct from the myeloid and lymphoid cells. Amongst the stromal cells, pericytes were most similar to fibroblasts, FRCs and DNs.

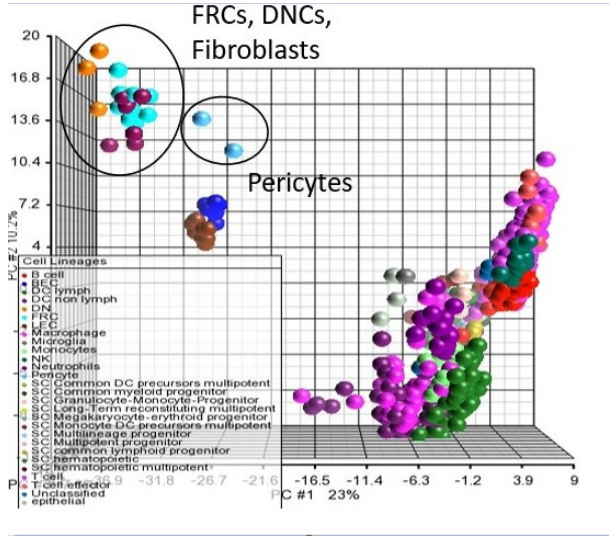

Figure 3: Principal Component Analysis (PCA) on the 681 cell lineages and two human pericytes samples using the vascular gene list of 384 genes. The pericytes samples (light blue, up-middle) cluster closely to specific stromal cells namely fibroblastic reticular cells (turquoise, up-left), double negative cells (orange, up-left) and fibroblasts (purple, up-left) indicating a shared expression profile.

Tolerance for Copy Number Variation (CNV) in stromal cells

Lastly, we studied gene cluster 5 to assess the tolerance and impact of large base pair alterations in stromal cells. We identified their Copy Number Variation (CNV) z-score, found by Exome Aggregation Consortium (ExAC). Some genes had no CNV and thus a CNV z-score=NaN (highlighted in Figure 2). These genes likely play a crucial role in (embryogenesis) pluripotent stem cells explaining the intolerance for loss-of-function mutations as these would be lethal. These genes were predominantly over-expressed in DNs and/or FRCs.

DISCUSSION

In this study, we performed a hierarchical clustering analysis with genes that have a causal link to vascular phenotypes by known genetic mutations in various databases using the immunological genome project RNA expression atlas. Here we describe the results that vascular genes are biased for overexpression in stromal and myeloid lineages. This illustrates the importance of these cell types in vascular morphogenesis. Moreover, we found a subset of genes with exclusive overexpression in stromal, mesenchymal cells that are closely related to pericytes. Functional pathway analysis of these genes shows enrichment in global pericyte functions, stressing the importance of pericyte stem cells in vascular development and maintenance. This is in line with other studies that have shown that pericytes show very similar RNA expression profiles as MSCs [29].

Pericytes promote the survival of endothelial cells and regulate vessel stabilization and limit vascular permeability. Disruption of stable pericyte-endothelial cell connections in tumors enables sprouting angiogenesis and creates a dysfunctional vascular network [9,30]. As another function of pericytes is the maintenance of the blood-brain barrier it might also explain the substantial amount of enriched pathways related to the nervous system and cancer in our IPA analysis. A clear hallmark of solid tumor formation is vascularization. Furthermore, genes expressed in stromal cells also shared expression with myeloid cells, in contrast to lymphoid cells. These genes might contribute to the observed direct association between perivascular macrophages and angiogenic blood vessel development [30,31]. However, our data demonstrate that not only macrophages, but also other myeloid cell types may influence vascular morphogenesis.

Our results are in line with studies of Infantile Hemangioma (IH) and may provide novel insights for personalized and targeted therapeutic options. In IH pluripotent Hemangioma Stem Cells (HemSC) cause these lesions and these cells may differentiate into endothelial cells, pericytes and adipocytes [32]. Moreover, MSCs play a crucial role in the pathogenesis of IH, where they reside in the perivascular region and mostly express the pericyte markers PDGFR–β and α–SMA [33]. Not only MSCs but also immunological cells such as macrophages contribute to the progression of IH by stimulating the proliferation and endothelial differentiation of HemSC, while suppressing adipogenesis [34,35]. This is further highlighted by the vascular comorbidities found in immunological syndromes such as PTEN hamartoma tumor syndrome [17]. Therefore, inflammatory cytokines and ERK1/2 and PI3K/AKT/PTEN pathway enhancement may be involved in this macrophage-induced proliferation, which may point to future therapeutic targets. For example, the mTOR pathway is closely related to the PI3K/AKT/PTEN pathway and mTOR inhibitor sirolimus (rapamycin) has been used in the treatment of patients with severe vascular malformations [36]. The PI3K pathway is upstream of mTOR and opens up the possibility of specific PI3K inhibition with agents like alpelisib [37].

Whilst analysing the gene functions, we identified that our vascular

gene set list highly enriched for pathways related to EMT (20%; 37/189 genes). EMT is integral to development and regulates embryonic stem cell differentiation and behaviour [38,39]. Similar to epithelial cells, endothelial cells can also transdifferentiate into mesenchymal phenotypes, referred as Endothelial-Mesenchymal-Transition (EndMT). Both EMT and EndMT transcription programs and signaling pathways are very similar [39,40]. Studies indicate that Snail and Slug, transcription factors of EMT and EndMT, mediate sprouting angiogenesis, particularly in pathological angiogenesis such as cancer [41]. Our findings suggest a possible link between EMT/EndMT and pericyte recruitment and/or differentiation. It is already known that EMT and EndMT can generate mesenchymal cells that express αSMA, a marker of pericytes and myofibroblasts [10,40,42]. However, there is also conflicting evidence, which suggests pericytes do not behave as mesenchymal stem cells *in vivo* [43]. Therefore, more validation studies are scheduled to further investigate the relation of EndMT and pericyte recruitment and or differentiation *in vivo*.

The major strength of our data mining approach is that we integrated different data sources to discover unidentified mesenchymal stromal cell functions, behavior and interactions of genes with roles in other stromal and immunological cell types. The formation of blood vessels is a highly integrated process requiring coordinated expression of hundreds of genes and proteins in different cell lineages [4,44,46]. To further study these genes and lineages, we used the highly detailed ImmGen RNA expression data [25]. Furthermore, IPA allows us to study the associated biology of known genetic mutations through revealing their consistent and highly significant pathways with clinical implications. In the past decade, numerous public databases have been generated through whole genome sequencing [26,27]. Our approach could be applied as an extensive analysis method to further investigate these databases and therefore discover new biology underlying different clinical entities.

There are also limitations in our analysis. First of all, pericytes are hard to identify as the molecular marker PDGFR–β to study pericyte cell functions is expressed in multiple cell lineages in embryology [9,43]. DNs in ImmGen are characterized as fibroblastic, contractile pericytes and stained anti-ITGA7 positive. However, to confirm our data analysis derived findings, further distinctions of pericytes from other peri-endothelial mesenchymal cells are needed. Our conclusions could therefore only be drawn for mesenchymal stromal cells in general. We have tried to parry these limitations by adding another dataset of pericytes where we validated our results. It would be interesting to add more data on other cell types like mesoangioblasts and other pericyte subtypes to further confirm and validate our results.

Moreover, we proposed the skin as target organ to perform future experiments with selected human genes known in causing skin vascular changes. However, the mouse gene selection was not limited to vascular defects of the skin. Lineage-tracing studies demonstrated that pericytes have different developmental origins and their functions and densities are tissue-specific [9,47,48]. Therefore, skin-vascular experimental models are needed to confirm whether the conclusions are applicable for the skin. For the selection of skin vascular genes, we used the POSSUM database instead of limiting to reported mutations of vascular anomalies. This could clarify why we only found 3% overlap between the human and mouse genes. However, some anomalies have mutations in more than one gene and vascular anomalies are often associated with (overgrowth) syndromes. Therefore, our method enabled us to discover and understand (new) gene functions related to vasculature. Furthermore, evidence for conservation of the immunological transcriptional program of human and mouse genes is variable in different cell lineages [49,50]. Thus, *in vitro* and in vivo analyses are needed to confirm whether our results are also applicable to human biology [51-56].

CONCLUSION

Our study improves the understanding of vascular development and morphogenesis and has several implications for future clinical studies applying NGS techniques. For example, our study might help design and select the appropriate cell type to perform RNA sequencing analyses concerning mutation detection within these genes. Our approach identified known mutations in causing vascular anomalies in humans and generated a new set of unidentified candidate genes and their stromal cell expressions. Most identified mutations of vascular malformations in humans were expressed in endothelial cells. Our results indicate that unknown mutations may show expression in mesenchymal cells/pericytes and are related to EMT/EndMT induced pathways. Pathways affected in the pathogenesis of vascular malformations, were also identified in IH which opens up the possibility for therapeutic interventions targeted at the molecular mechanism. In future, vascular anomalies need to be further histochemically investigated for the presence and function of MSCs and the expression of transcription factors and markers of EMT/EndMT as these might pose potential therapeutic targets.

AUTHORS CONTRIBUTIONS

Liu Liu and Rogier T. A. van Wijck contributed equally to the manuscript and are co-first authors

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