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Down Syndrome Expressed Protein; DSCR-1 Deters Cancer and Septic Inflammation

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1. Introduction

Down syndrome is the most common genetic cause of mental retardation in humans, occurring in one out of 700 live births. Epidemiological studies suggest that although individuals with Down syndrome have an increased risk of infant cardiovascular malformation, muscle hypotonia, lymphatic edema, and leukemia, noteworthy they have a considerably reduced incidence of most solid tumor, atherosclerosis, and pathological angiogenesis-mediated diabetic retinopathy and kidney dysfunction.

Such data indicate that one or more of the 231 trisomic genes on chromosome 21 are responsible for protecting these individuals against cancer and vascular disease. We and others recently have identified the candidate genes are Down syndrome critical region (DSCR)-1, and A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-1. In primary cultured endothelial cells, vascular endothelial cell growth factor (VEGF) resulted in rapid and profound upregulation of both genes, which in turn negatively feeds back to attenuate VEGF-mediated signaling and following the endothelial cell activation. In genome-wide screening, important regulatory transcription factor for many pathological features of Down syndrome, NFAT, bound more than 10,000 independent regions in VEGF-treated activated endothelial cells. Down syndrome trisomy model mice or endothelium-specific modest DSCR-1 increases in mice resulted in significant suppression of the vascular density in matrigel-plugs, inflammatory leukocyte infiltration, and tumor growth. In contrast, DSCR-1 null mice demonstrated markedly decreased vascular integrity and increased susceptibility to tumor metastasis. In a mouse model of endotoxemia, DSCR-1 null mice showed greater morbidity and mortality compared with wild-type littermate. Conversely, adenovirus-mediated overexpression of DSCR-1 resulted in marked attenuation of lipopolysaccharide (LPS) or VEGF-mediated inflammation. Collectively, these data provide that Down syndrome overexpressed protein; DSCR-1 serves to dampen the host response to infection and the tumor growth. The molecular research for Down syndrome with patients or model mice unexpectedly provide us a great hint for therapeutic targets in solid tumor and vasculopathic disease against all individuals.

2. Down Syndrome Critical Region (DSCR)-1 expression in activated endothelium

2.1 Foundation of the DSCR-1 from endothelial cell research

The endothelium is highly malleable cell layer, constantly responding to changes within the extracellular environment and responding in ways that are usually beneficial, but at times harmful to the organism. Several mediators, including growth factors (e.g. vascular endothelial growth factor, VEGF), inflammatory cytokines (e.g. tumor necrosis factor-α, TNF-α), and thrombosis mediator (e.g. thrombin), activate gene transcription in endothelial cells, resulting in changes in hemostatic balance, increased leukocyte adhesion, loss of barrier function, increased permeability, migration, proliferation and successive angiogenesis (Minami and Aird, 2005). The tight control of these processes is essential for homeostasis - endothelial cell activation, if excessive, sustained or spatially and temporally misplaced, may result in vasculopathic disease. Indeed, different extra-cellular mediators engage the endothelium in ways that differ from one signal to the next. A major important point is to survey the temporal and spatial dynamics of endothelial cell activation. Using DNA microarrays, I carried out a global survey of mRNA in human umbilical vein endothelial cells (HUVEC) treated in the VEGF, thrombin, or TNF-α. Clustering analyses of the data revealed a far closer relationship between VEGF and thrombin, than between other pairings (Fig. 1A). Of the various transcripts that were responsive both to VEGF and thrombin, DSCR-1 was the most highly induced at the earliest time point (1 h). Compared with VEGF and thrombin, TNF-α treatment of HUVEC resulted in far less induction of DSCR-1 (3.2-fold at 1 h) (not shown). The rest of the VEGF-mediated induced gene was early growth response (Egr)-3, nerve growth factor inducible (NGFI)-Bβ, cyclooxigenase (COX)-2, and ADAMTS-1 (**Fig. 1B**).

2.2 Molecular information of the DSCR-1

The DSCR-1 gene consists of 7 exons, of which exons 1-4 can be alternatively spliced, resulting in a number of different mRNA isoforms, each of which exhibit different expression patterns. In adult, there are two major isoforms, DSCR-1 long variant (DSCR-1L) and DSCR-1 short variant (DSCR-1s), expressed in organs (Fuentes et al., 1997). DSCR-1L, encoded by exons 1, 5, 6, and 7, is highly expressed in brain. Exon 1 was originally thought to encode a 29 amino acid region, but later studies revealed a start site further upstream, resulting in a larger 84 amino acid region (Genesca et al., 2003). In contrast, DSCR-1s is encoded by exons 4-7 and is under the control of a different promoter located in intron 3 (intergenic promoter) (**Fig. 1C**). Each promoter contains different regulatory transcriptional subunits. For example, DSCR-1s is mainly regulated by the calcineurin-NFAT pathway, which is highly induced by angiogenic and inflammatory stimuli in endothelial cells (Minami et al., 2004; Minami et al., 2006).

While, the DSCR-1L isoform is under the control of a Notch and Hes-1-dependent pathway (Mammucari et al., 2005) or TEF-1 dependent pathway (Liu et al., 2008). DSCR-1s inhibits calcineurin phosphatase activity, and the C-terminal 57 residues are sufficient for this activity. DSCR-1s strongly inhibits the calcineurin mediated NFAT signaling via two ways; its ability to disrupt binding of calcineurin to NFAT, and to disrupt calcineurin enzymatic activities (**Fig. 1D**).

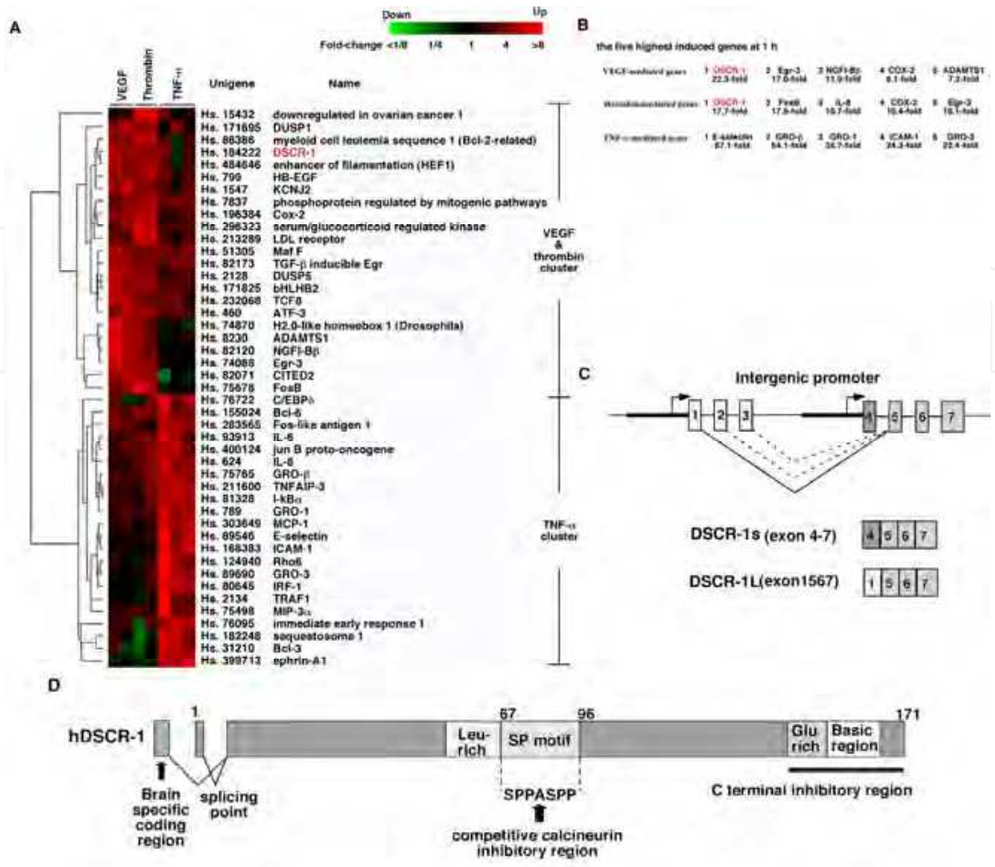

Fig. 1. VEGF / thrombin-mediated DSCR-1 induction *A*, heat map representation of induced genes in HUVEC. *B*, Top 5 genes induced via VEGF, thrombin, or TNF-α. *C*, major two variants of DSCR-1. *D*, structure information of DSCR-1

2.3 DSCR-1 expression in cultured cells

VEGF or thrombin induces the DSCR-1s expression in endothelial cells, through the coordinate binding of NFATc and GATA to closely positioned NFAT and GATA motifs in the intergenic promoter (Minami et al., 2004). VEGF/thrombin induces NFATc nuclear localization, and overexpression of the nuclear NFATc1 greatly induces the targeted DSCR-1s expression (Hesser et al., 2004; Minami et al., 2004; Minami et al., 2006). In addition, endothelial cells from the Down syndrome model mice (Ts65Dn) increased DSCR-1 mRNA by 1.7-2.0 fold (Baek et al., 2009). NFATc is an important factor for regulating the vertebrate development (Graef et al., 2001). In endothelial cells, NFATc1, c2, and c3 are expressed (Minami et al., 2009). To survey the NFATc1 binding in genome-widely, we carried out the chromatin immunoprecipitation using the antibody against NFATc1 following the comprehensive sequencing (ChIP-seq) in endothelial cells. We found totally 10,938 regions (*P* value >20) were identified as NFATc1 enrichment area from the ChIP-seq. DSCR-1 revealed the profound NFATc1 binding after the VEGF treatment within the proximal DSCR-1s promoter (**Fig. 2**). The area overlapped with positive signals from acetylated histone H4 (transcriptional active chromatin) and tri-methylated lysine of histone H3 (H3K4me3; active promoter marking) (**Fig. 2**).

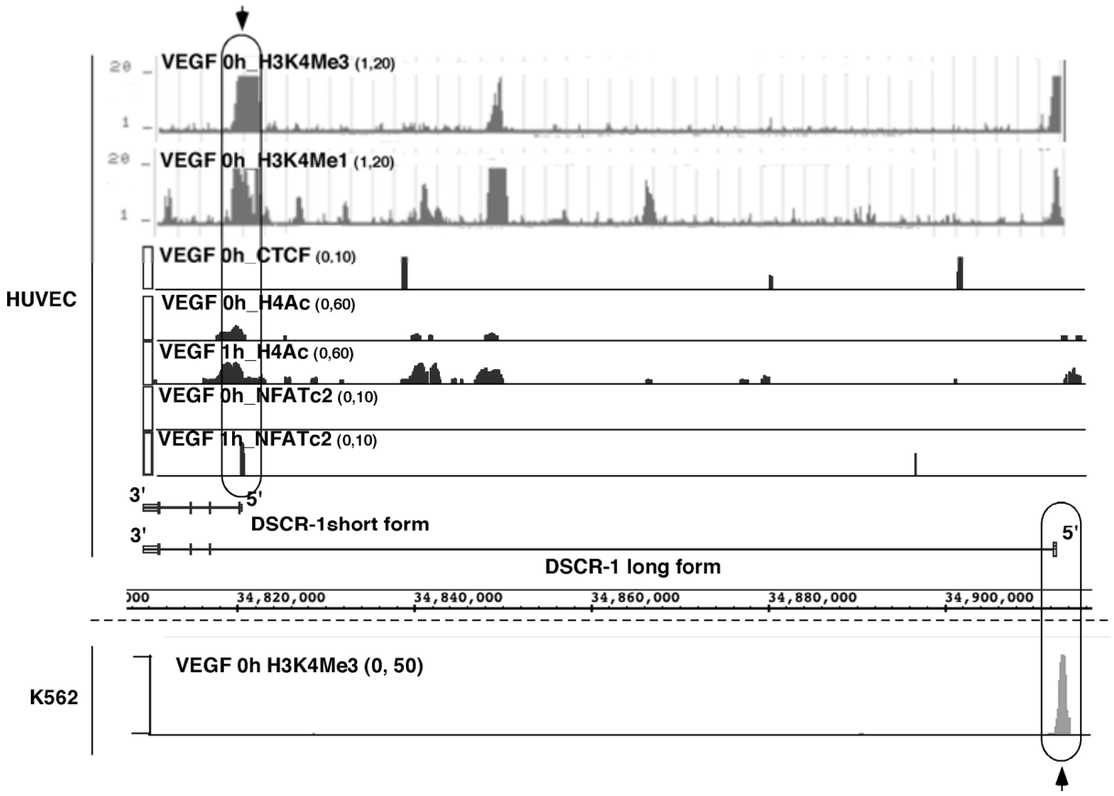

Fig. 2. Epigenetic information of the DSCR-1 locus Arrow indicates the significant enrichment from the ChIP-seq results

In contrast, erythroid lineage cells isolated from leukemia; K562 indicated the H3K4me3 positive signals within the proximal DSCR-1L promoter region, but not proximal DSCR-1s promoter region (**Fig. 2**). DSCR-1L reported the proceeding the pathological function in neurons (Cook et al., 2005). Moreover, Down syndrome patients have an increased risk of leukemia (Lott, 1982). Collectively, DSCR-1s and DSCR-1L obtained separate transcriptional machinery. VEGF mediated NFATc activation selectively transactivates the DSCR-1s via the profound binding within the promoter.

2.4 Characterization of the NFAT dependent genes overexpressed in Down syndrome

Besides DSCR-1, other genes encoded in chromosome 21 also reported as a candidate for pathogenesis on the Down syndrome. By using the combination of several NFATc knockout mice, dysfunction of NFAT was shown as a key point for the onset of Down syndrome (Arron et al., 2006). Around 1.5-fold increasing of both DSCR-1 and DYRK1A caused complete NFAT dysfunction. Thus, we test whether many Down syndrome genes obtain the NFATc1 binding on the each proximal promoter, by using the whole-genome NFATc1 ChIP-seq data (**Table 1**). Interestingly, DYRK1A obtained positive NFATc1 binding. VEGF inducible ADAMTS-1 (see **Fig. 1B**) also showed the NFATc1 positive binding. Ets family, Ets2, ERG, and GABPα, were highly expressed in endothelial cells, which was shown the regulation for the endothelial cell-specific expression or-essential function. All of them have a possibility as a NFATc1 direct target downstream gene.

	Gene	Description	NFATc1 bound	Bound peak area
From Nature (Reynolds et al.2010)				
	ADAMTS1	A disintegrin and metalloproteinase with thrombospondin motifs, type 1	+	5′UTR
	ERG	v-ets erythroblastosis virus E26 oncogene homolog	+	+5367 & 5th intron
	Ets2	v-ets erythroblastosis virus E26 oncogene homolog 2	+	+55
	JAM2	junction adhesion molecule 2	-	
	PTTG1IP	pituitary tumor-transforming 1 interacting protein	+	+883
From Lancet (Roizen and Patterson, 2003)				
Energy and reactive oxygen species metabolism				
	BTG3	B-cell translocation gene 3	+, weak	1st intron
	MRPL39	mitochondrial ribosomal protein L39	+, weak	1st exon
	ATP5J	ATP synthase, H+ transporting, subunit F6	+, weak	1st exon
	GABPA	GA binding protein transcription factor, alpha	+, weak	+80
	BACH1	BTB and CNC homology 1, basic leucine zipper transcription factor 1	+, weak	1st intron
	SOD1	superoxide dismutase 1	+	5′UTR
	CRYZL1	crystallin, zeta-like 1	+, weak	1st Exon & +520
	ATP5O	ATP synthase, H+ transporting, O subunit	+	5′UTR
	MRPS6	mitochondrial ribosomal protein S6	+, weak	5'-UTR
	DSCR-1	Down syndrome critical region gene 1	+	Indicated in Fig. 2
	CBR1	carbonyl reductase 1	+, weak	1st exon
	CBR3	carbonyl reductase 3	+	1st exon
	SH3BGR	SH3 domain binding glutamic acid-rich protein	+	5′UTR
	NDUFV3	NADH dehydrogenase flavoprotein 3, 10kDa	+	+139
	SNF1LK	salt-inducible kinase 1	-	
	C21orf2	chromosome 21 open reading frame 2	+, weak	+140
Brain development, neuronal loss, and Alzheimer's type neuropathology				
	SIM2	single-minded homolog 2	-	
	DYRK1A	dual-specificity tyrosine-phosphorylation regulated kinase 1A	+	+1680

	GART	phosphoribosylglycinamide formyltransferase	+	+513 & 5'-UTR
	PCP4	Purkinje cell protein 4	-	
	DSCAM	Down syndrome cell adhesion molecule	-	
	GRIK1	glutamate receptor, ionotropic, kainate 1	-	
	APP	amyloid beta (A4) precursor protein	+, weak	1st intron
	S100B	S100 Ca-binding protein B	-	
Folate methyl group metabolism				
	N6AMT1	N-6 adenine-specific DNA methyltransferase 1	+, weak	1st exon
	CBS	cystathionine-beta-synthase	-	
	DNMT3L	DNA methyltransferase 3-like	-	
	SLC19A1	Solute carrier family 19 , member 1	-	
	FTCD	formiminotransferase cyclodeaminase	-	
	HRMT1L1 (PRMT2)	Protein arginine methyltransferase 2	+	5′UTR

Table 1. Candidate genes for Down syndrome, where NFATc1 occupancy on the promoter. 'Weak 'means *P* value < 20

2.5 DSCR-1 expression in organ

Increased DSCR-1 expression was observed in human fetal Down syndrome kidney versus age-matched control kidney (**Fig. 3A**). To determine whether the DSCR-1s promoter region directed inducible expression *in vivo*, the -1664/+83 DSCR-1s promoter was coupled to the *lacZ* reporter gene and targeted the resulting transgenic cassette (DSCR-1-*lacZ*) to the *Hprt* locus of mice using homologous recombination. The *Hprt*-locus *in vivo* promoter analysis system has been previously shown to be beneficial in controlling and avoiding the undesirable and undetectable effects of copy number and integration site on promoter activity (Cvetkovic et al., 2000; Ryan and Sigmund, 2003). We have used this system successfully to show the vascular bed specific expression patterns of endothelial cell specific promoters, Flt-1, vWF, ROBO4, and Tie-2 (Minami et al., 2002; Minami et al., 2003; Okada et al., 2007). At embryonic day 11, whole-mount *lacZ* staining revealed widespread expression of the transgene in the vasculature. In cryosections, strong staining was observed in the dorsal aorta, intersomitic vessels, carotid arteries, caudal veins, the primary head vein branch, and the endocardium (**Fig. 3B**). *LacZ* colocalized with endothelial PECAM-1 (**Fig. 3B**). However, after the embryonic day 14, profound DSCR-1s promoter activation in vascular endothelium was markedly downregulated correlated with the decline of embryonic VEGF levels after the critical steps for angiogenesis and vascular remodeling. In adult mice, DSCR-1s-*lacZ* activity was detected in only a subset of endothelial cells in the brain, heart, lung and kidney. Expression was also observed in occasional neurons, vascular smooth muscle cells, cardiomyocytes, and renal epithelial cells. In contrast, DSCR-1s-*lacZ* activity was undetectable in the liver, spleen, thigh skeletal muscle, and thymus. These findings suggest that the DSCR-1s promoter, though widely expressed in the endothelium of embryonic days 11, is downregulated in the later stages of development and in adults.

Subsequently, to determine whether the DSCR-1s promoter confers response to inflammatory or angiogenic stimuli *in vivo*, DSCR-1-*lacZ* mice were systemically administrated VEGF or LPS. In whole mount preparations, the X-gal reaction product was detectable in the brain and heart of untreated mice and was further upregulated by VEGF and LPS. In contrast, *lacZ* staining was not observed in skeletal muscle, liver, and spleen vasculature even after the stimulus (data not shown).

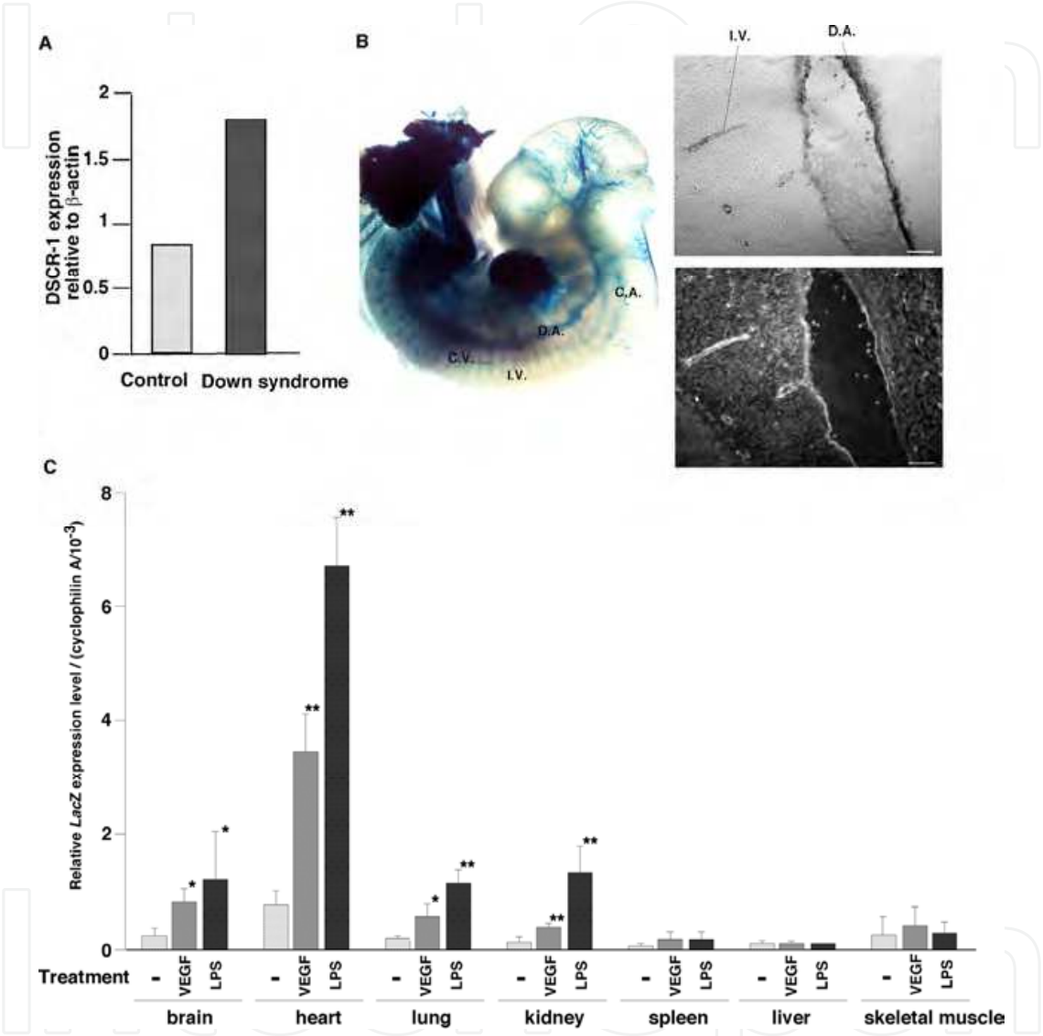

Fig. 3. DSCR-1 promoter activation *in vivo A,* highly DSCR-1 expression in kidney from Down syndrome individuals. *B, left*, whole-mount *lacZ* staining of embryonic day 11 Hprt-targeted embryos. C.A., carotid artery; D.A., dorsal aorta; I.V., intersomite vessel. *Right,* serial tissue sections from Hprt-targeted embryo. *lacZ* (*up*) and PECAM-1 (*down*) immuno-stainings were shown. *C,* real-time PCR quantification of *lacZ* epression in various organs. *P<0.04, **P<0.01 compared mock treatment in each organ.

Real-time PCR analysis was used to quantify changes in transgene expression. Under basal conditions, *lacZ* mRNA expression was highest in the heart, followed by the brain, lung, and kidney (**Fig. 3C**). Expression in skeletal muscle, the spleen and liver was below the level of detection. VEGF and LPS resulted in significant induction of *lacZ* transcripts in the heart,

brain, lung, and kidney, but not in spleen, liver or skeletal muscle (**Fig. 3C**). LPS-mediated induction of the endogenous DSCR-1s gene was similarly restricted to the heart (25.3-fold), brain (7.0-fold), lung (10.3-fold), and kidney (9.3-fold) (not shown). Agonist treatment failed to alter DSCR-1L transcript levels. Thus, VEGF and LPS promote vascular bed-specific expression of both the DSCR-1s promoter and the endogenous DSCR-1s gene.

2.6 DSCR-1 expression in tumor

Solid tumors produce a variety of pro-angiogenic molecules and inflammatory cytokines, which have important paracrine effects on surrounding endothelial cells. To investigated whether the DSCR-1s transgene is activated in tumor blood vessels, B16-F1 melanoma and Lewis lung carcinoma (LLC) cells were implanted subcutaneously into the flank of DSCR-1s-*lacZ Hprt* mice. When tumors reached ≈2.5 cm^3 in volume, the xenografts were harvested, sectioned and stained for *lacZ*. As shown in **Fig. 4A**, there was widespread reporter gene activity within both B16-F1 melanoma and LLC tumor neovessels. In double immunofluorescence studies, *lacZ* co-localized with endothelial PECAM-1 (**Fig. 4B**). Consistent with these findings, endogenous DSCR-1 also co-localized with PECAM-1 in tumor vessels of both B16-F1 melanoma and LLC xenografts (not shown).

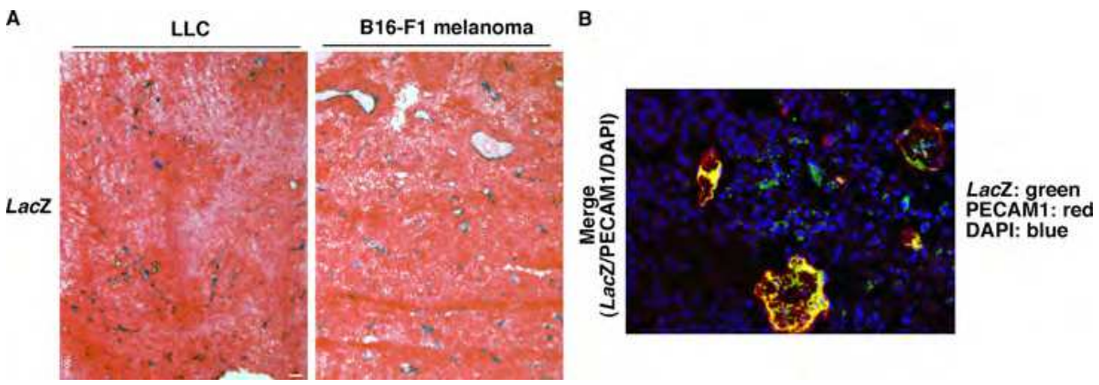

Fig. 4. DSCR-1s promoter activation in tumor vasculature. *A, lacZ* stainings were performed of LLC and B16-F1 melanoma xenografts. *B,* merged image of immunofluorescence staining with antibodies against *lacZ*, PECAM-1 or DAPI.

3. Biological function of DSCR-1

3.1 DSCR-1 inhibits nuclear localization of NFATc

Adenovirus mediated overexpression of DSCR-1, but not control, inhibited VEGF mediated nuclear localization of NFATc1 and NFATc2 (Minami et al., 2004). DYRK1A is another potential NFAT regulators, which encodes a nuclear serine/threonine kinase that primes substrates for phsphorylation by Glycogen synthase kinase (GSK) 3(Gwack et al., 2006). GSK3 phosphorylates NFATc proteins in the nucleus, resulting in their inactivation and export (Beals et al., 1997). DYRK1A is expressed at elevated levels in some human Down syndrome fetal tissues (Arron et al., 2006). In neuronal cells, DYRK1A inhibits FGF8-mediated induction of NFAT activity. Moreover, it has been shown that a 1.5-fold increase in the dosage of DSCR-1 and DYRK1A, both of which lie within the critical region of human chromosome 21, cooperatively destabilized the calcineurin-NFAT regulatory circuit (Fig. 5), leading to many of the features of Down syndrome (Arron et al., 2006).

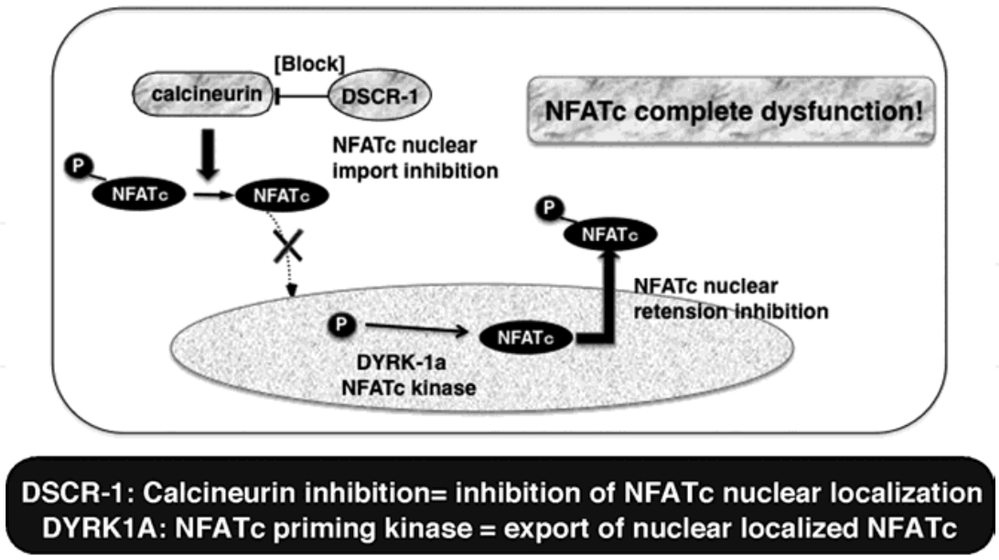

Fig. 5. schematic model of NFAT inhibition with DSCR-1 and DYRK1A.

3.2 DSCR-1s auto-inhibits the VEGF-mediated vascular activation

VEGF is an endothelial cell specific mitogen, and chemotactic agent, which is involved in wound repair, angiogenesis of ischemic tissue, tumor growth, microvascular permeability, hemostasis and endothelial cell survival (Isner and Losordo, 1999). DSCR-1 overexpression inhibits VEGF-mediated vessel growth, and monocyte cell adhesion (Minami et al., 2006). DSCR-1 overexpression did not lead in increased apoptosis (Minami et al., 2009). Taken together, these findings suggest that DSCR-1 constitutive expression lead the endothelial cells to quiescent status form the VEGF-mediated activated status.

3.3 DSCR-1s attenuates septic inflammation

As shown above, DSCR-1s attenuates VEGF-mediated activation of cultured endothelial cells. These data led us to hypothesize that VEGF- and LPS-inducible expression of DSCR-1s in mice may serve as a negative feedback inhibitor of endothelial activation *in vivo*. To test this hypothesis, I examined the effect of DSCR-1 deficiency or overexpression on endotoxemia phenotype. The generation of DSCR-1$^{-/-}$ mice, which carry a targeted deletion of both DSCR-1s and DSCR-1L. To overexpress DSCR-1s, I have chosen an adenoviral delivery system in which the endothelial-specific Flt-1 promoter is coupled to DSCR-1s cassette (Ad-Flt1-DSCR-1s). *In vivo* delivery of Ad-Flt1-DSCR-1s results in overexpression of DSCR-1s in the intact endothelium of mice. Endotoxemia in mice is associated with a reduction in heart rate, blood pressure, and body temperature, and an increase in circulating interleukin (IL)-6 levels. This effect was accentuated in DSCR-1$^{-/-}$ mice, and attenuated in DSCR-1s overexpressing animals. Recently, Yano et.al. reported that endotoxemia in mice is associated with increased circulating levels of VEGF (Yano et al., 2006). Further, VEGF plays a pathogenic role in sepsis (Yano et al., 2008). Interestingly, resting levels of plasma VEGF were 5.0-fold higher in DSCR-1$^{-/-}$ mice compared with wild-type littermates (**Fig. 6A**), which is a parallel correlation with the report that lower VEGF expression in stem cell culture derived from amniotic fluid in Down Syndrome (Salvolini et al., 2010). In response to septic treatment, DSCR-1$^{-/-}$ mice demonstrated super-induction of circulating VEGF levels (2.2-

fold higher vs. wild-type mice). Ad-Flt1-DSCR-1s-injected mice had no change in resting VEGF levels. However, in response to endotoxemia, DSCR-1s-overexpressing mice demonstrated a 61% reduction in circulating VEGF levels compared with septic Ad-Flt1-control (**Fig. 6A**).

To assay for endothelial activation, real-time PCR was performed to measure mRNA expression of E-selectin, intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 in tissues from mice 6 h following injection of PBS (control) or LPS. Compared with wild-type littermate controls, LPS-treated DSCR-1$^{-/-}$ mice demonstrated super-induction of E-selectin in the heart and lung, ICAM-1 in heart, and VCAM-1 in lung. In contrast, LPS-mediated induction of cell adhesion molecules was attenuated by overexpression of DSCR-1s (data not shown).

I have recently shown that hyper-activation of the VEGF-calcineurin-NFAT pathway triggers apoptosis in DSCR-1-deficient tumor endothelial cells (Minami et al., 2009). Given that DSCR-1$^{-/-}$ mice have elevated circulating levels of VEGF level (see **Fig. 6A**), I hypothesized that endotoxemia may result in increased endothelial cell apoptosis in DSCR-1$^{-/-}$ mice. To test this hypothesis, TUNEL assay was carried out in tissue sections from the heart and lung of LPS-treated DSCR-1-null mice and their wild-type littermates. Endotoxemic wild-type mice demonstrated a small number of TUNEL-positive endothelial cells in the heart, and even fewer in the lung. However, in DSCR-1$^{-/-}$ mice, LPS administration resulted in a significant increase in the number of TUNEL-positive cells in both organs (Minami et al., 2009).

Finally in survival studies, LPS-treated DSCR-1$^{-/-}$ mice demonstrated markedly increased mortality compared with endotoxemic wild-type littermates (**Fig.6B**, *left*). In contrast, Ad-mediated overexpression of DSCR-1s conferred a survival advantage compared with Ad-Flt1-control (**Fig. 6B**, *right*). Taken together, these findings suggest that inflammatory induced DSCR-1s obtains a critical role in the host response.

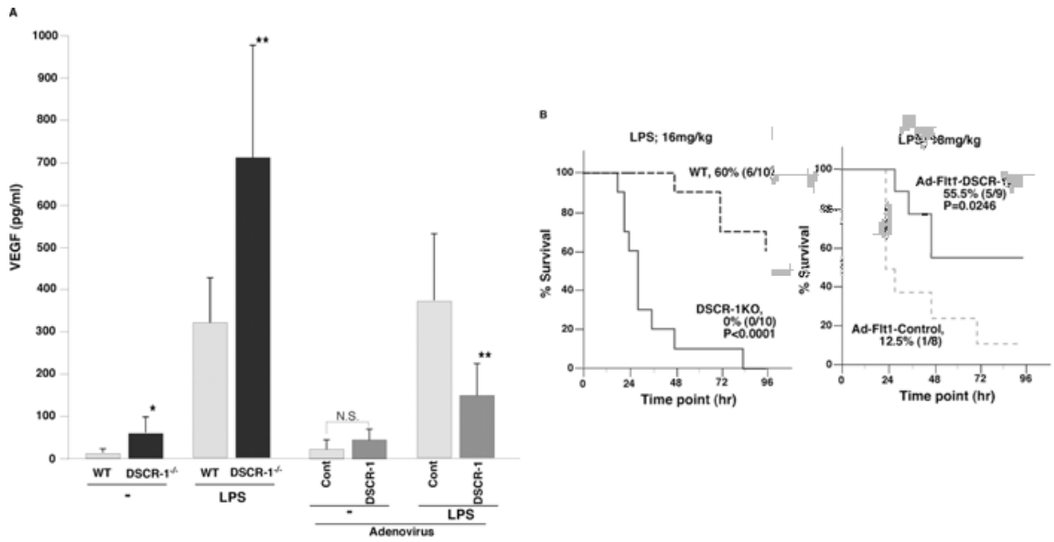

Fig. 6. DSCR-1 modulates inflammatory VEGF expression and sepsis mortality. *A*, Blood was harvested for plasma, and VEGF levels were measured using ELISA. *P<0.001, **P<0.01 compared with wild type or Ad-Flt1-control (cont), n=10. *B*, Survival studies in mice administrated LPS. Percentages of mice (surviving/total) are indicated.

3.4 DSCR-1s attenuates tumor progression

Having established an inhibitory role for DSCR-1 on inflammation *in vivo*, I next study the functional relevance of angiogenesis, tumor growth, and tumor metastasis *in vivo*. Adenovirus mediated locally DSCR-1 expression in vascular demonstrated significant reduction of the blood vessel formation in a matrigel-plug, compared to the Ad-control treated-plug vascularity. In a xenograft model with B16 melanoma and Lewis lung carcinoma revealed that DSCR-1 overexpression statistically significant (more than 70%) reduction of the tumor growth, in the parallel for the reduction of the vessel density from DSCR-1 treated tumor. Subsequently, to test whether DSCR-1 stable expression in the vascular endothelium inhibit the tumor growth, the transgenic mice was generated containing the endothelial cell specific Tie 2 promoter-linked DSCR-1s cDNA constract. Two independent transgenic lines indicate the vascular-specific DSCR-1s expression, both of which delayed the tumor growth at the early step, up to the tumor mass ≈1,500 cm^3. Cryosection of the xenografted tumor and immunostained with anti-PECAM1 antibody revealed the reduction of the vascular density in vascular specific DSCR-1s transgenic mice compared with wild type control mice. Taken together, DSCR-1s expressed in endothelial cells *in vivo* would function as an anti-angiogenic molecule. Stable expression of the DSCR-1s would lead the endogenous anti-tumor activities.

3.5 DSCR-1s attenuates tumor metastasis

During the study for the DSCR-1s promoter activity *in vivo*, by using the tumor metastasis model, we found the DSCR-1s promoter was already active in the lung microvascular endothelium, before the tumor metastasis colony had not yet observed. This DSCR-1s-*lacZ* activity in endothelial lining of lung was clearly abolished by systemic treatment with cyclosporine A. In addition, we have previously shown that stimuli induced DSCR-1 auto-inhibited inflammation in HUVEC (Minami et al., 2004; Minami et al., 2006). Thus, I hypothesized two things; DSCR-1s promoter would be useful for the marker for the pre-metastatic condition, and the DSCR-1s stable expression would overcome the tumor metastasis. At first, to test the latter thing, mice were injected intravenously with $2x10^5$ B16 melanoma cells. Three days later, mice were administrated with adenovirus containing Flt-1 promoter-DSCR-1s, or the control into the lung via the airway with vapor infection. Twenty days after the B16-F10 injection, lungs were harvested and photographed. There was no difference in body weight between the experimental and control groups (data not shown). The Ad-Flt1-Control group exhibited significant endothelial cell surface invaded-melanoma metastasis and the lungs showed significant swelling (**Fig. 7A**). In contrast, the Ad-Flt1-DSCR-1s group exhibited little B16 melanoma metastasis and showed no significant lung swelling. To semi-quantify the metastasis rate, the melanoma-growing area per whole surface was calculated. Compared with control, Ad-Flt1-DSCR-1s treatment resulted in statistically significant reduction (54%) of the B16 melanoma metastasis (**Fig. 7B**). In addition to DSCR-1s's ability to inhibit B16 melanoma metastasis to lung, DSCR-1s treated lung showed a significant reduction in the expression of the inflammatory adhesion molecules, VCAM-1 and E-selectin. It has been reported that endothelial cells expressed VCAM-1 and E-selectin positively influence cancer cell adhesion and migration to lung (Biancone et al., 1996; Fukuda et al., 2000; Futakuchi et al., 2004). Our performed metastasis assays, however, could not distinguish whether reduction in these adhesion molecules attenuated tumor metastasis, or reduced tumor cell migration to lung inhibited

inflammation and adhesion molecule induction. We have reported DSCR-1s strongly attenuates adhesion molecule expression in cultured endothelial cells (Minami et al., 2006). Moreover, DSCR-1 stably expressed in cultured endothelial cells significantly blocked B16 melanoma attachment to the endothelial cell surface (data not shown), suggesting that the blunting of adhesion molecule expression by DSCR-1s might be a critical factor in the inhibition of tumor metastasis. Collectively, These data suggest that DSCR-1s stable expression inhibits the inflammatory coordinated pre-metastatic niche formation, resulting the strong interfering of the onset of tumor metastasis to lung.

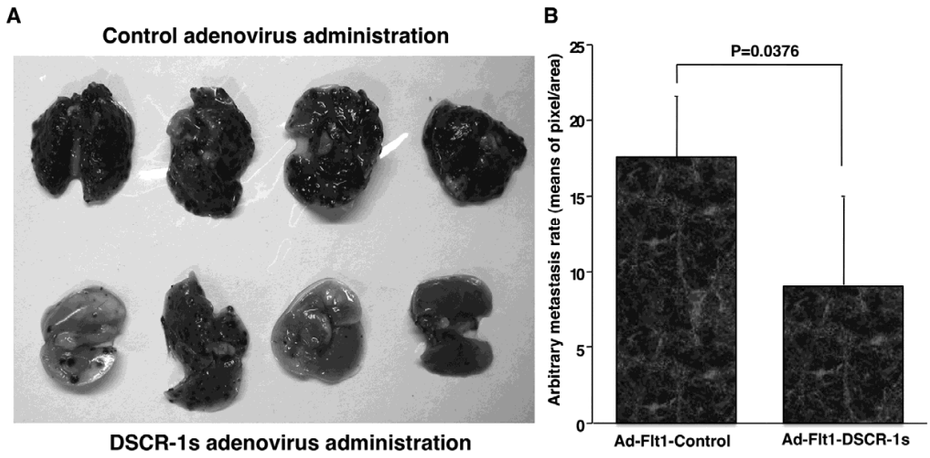

Fig. 7. Endothelial specific DSCR-1s overexpression downregulates the tumor metastasis to lung. *A,* after 20 days for B16-F10 injection administrated either Ad-Flt1-control or Ad-Flt1-DSCR-1s, lungs were harvested, washed and photographed. *B,* to quantify the metastasis rate, metastasis area from the whole surface lung was calculated by using Image J software. Data are expressed as means and standard deviations; n=10.

3.6 Lacking DSCR-1 results with controversy

While our findings reported here lend further evidence toward DSCR-1s as a negative regulator of NFAT-calcineurin signaling *in vivo*, the exact function of DSCR-1 is not without controversy. It has been reported that in CHO cells cultured *in vitro*, a portion of the phosphorylated form of DSCR-1 associated with the 14-3-3 protein, which competitively activated calcineurin activity (Abbasi et al., 2006). However, in a separate report it was shown that phosphorylation of DSCR-1 markedly decreased its stability (Genesca et al., 2003), likely leading to degradation, and thus an increase in calcineurin activity. In endothelial cells, we observed that the non-phosphorylated form of DSCR-1, which we consider to be the pre-active form, was the dominant form during the early phase response to VEGF or thrombin. The phenotype exhibited by the DSCR-1 whole gene knockout mice shows exacerbated constitutively active calcineurin-dependent cardiac hypertrophy, whereas cardiac hypertrophy in response to pressure overload and chronic adrenergic stimulation was blunted in these mice (Vega et al., 2003). In addition, double knockout mice of DSCR-1 and modulator of calcineurin interacting protein (MCIP) 2 also resulted in calcineurin facilitation, although it is difficult to distinguish the relative contribution between DSCR-1 and MCIP2 in these events (Sanna et al., 2006). It light of previous results and the results presented here suggesting differing expression patterns and functions of the DSCR-1s and 1L isoforms, it is plausible that the phenotype of the DSCR-1 null mice results

from the complex deletion of both DSCR-1L and DSCR-1s isoforms. Qin et.al., recently reported DSCR-1s inhibited vascular growth and capillary tube formation, consistent with our findings, whereas DSCR-1L induced NFATc transcriptional activity and endothelial cell growth (Qin et al., 2006). Using siRNA or adenoviral miRNA in endothelial cells or in mice *in vivo*, respectively, I recently showed that the DSCR-1s promoter is specifically activated through NFATc1, c2, c3 and GATA-2 (Minami et al., 2009), whereas DSCR-1L, which lacks the NFAT consensus region, is regulated by Notch and glucocorticoid signaling (Mammucari et al., 2005). The lack of such elements in the DSCR-1L promoter, suggest that once DSCR-1L is expressed, the tightly regulated calcineurin-NFATc-DSCR-1s feedback loop may be broken, owing to a lack of NFATc regulation of the DSCR-1L promoter. I also cannot rule out the possibility that the DSCR-1 exon 1 has an as yet 'undetermined' function. Interestingly, in an attempt to understand this regulatory system, an attractive computationally simulated threshold model was shown by Shin, et. al (Shin et al., 2006), in which low-level stimulus causes weak NFAT activation, resulting in only minor DSCR-1s upregulation, and at a level not sufficient to block the target calcineurin (under the threshold). Continuing stimulus facilitates the dissociation of the calcineurin-DSCR-1 complex, so that DSCR-1 appears as an activator of calcineurin.

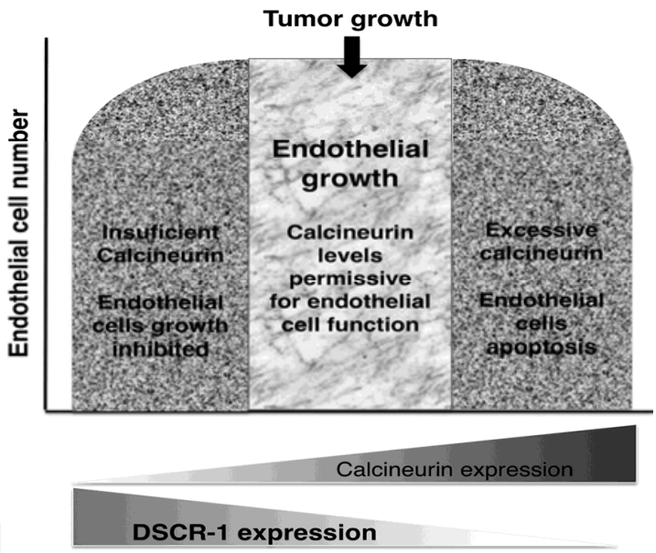

Fig. 8. Schmatic model of the balance of the endothelial cell growth

Interestingly in the endothelial cells, Sandra.et.al., indicated that DSCR-1$^{-/-}$ mice demonstrated reduced blood vessel formation in Matrigel, cornel micropocket, and tumor xenograft assays (Ryeom et al., 2008). DSCR-1$^{-/-}$ endothelial cells displayed hyper-activation of the calcineurin/NFAT pathway and increased sensitivity to VEGF signaling. However, rather than inducing cell proliferation, VEGF-mediated activation of calcineurin/NFAT in DSCR-1$^{-/-}$ endothelial cells 're-routed' downstream signaling, resulting in increased apoptosis, which thus explains the paradoxical reduction in neovascularization. Collectively, considered with the data from DSCR-1 stable expression and null mutation, calcineurin/NFAT activity and DSCR-1s expression level was tightly regulated, resulting the balance would define the endothelial cell growth, viability and tumor angiogenesis (**Fig. 8**). Future animal studies of DSCR-1 function should be performed by endothelial cell-specific knockout mice targeting either DSCR-1s or DSCR-1L separately.

4. Conclusion

DSCR-1 was identified by the study with vascular activation. DSCR-1 was highest induced by VEGF treatment in primary cultured endothelial cells. Previously, DSCR-1 was simply termed by the localization of the human chromosome 21. However, DSCR-1 indeed highly expressed in Down syndrome individuals, and clearly upregulated with NFAT activation in cells. Moreover, combined with same 21st chromosome encoded protein; 'DYRK1A', DSCR-1 strongly feedback attenuated the NFAT activation, resulting the pathogenesis of Down syndrome. I show here that DSCR-1s is highly expressed during embryonic vascular development, and then largely downregulated in adult, yet was highly activated predominantly in endothelium in response to the administration of VEGF or LPS. Stimulated DSCR-1s worked in the auto-inhibition of endothelial cell activation and inflammation. It has still unanswered problems with understanding the phenotypes from DSCR-1 lacking condition, and pathogenesis from DSCR-1L overexpression in neuron. However, based on this knowledge, I believe that DSCR-1s stable expression or the way of DSCR-1s stabilization may lend itself to therapeutic manipulation in vasculopathic disease states, including tumor angiogenesis, metastasis, and inflammation.

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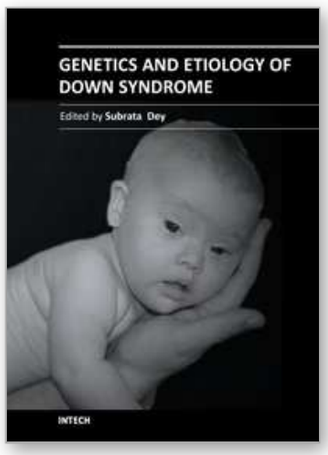

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This book provides a concise yet comprehensive source of current information on Down syndrome. Research workers, scientists, medical graduates and paediatricians will find it an excellent source for reference and review. This book has been divided into four sections, beginning with the Genetics and Etiology and ending with Prenatal Diagnosis and Screening. Inside, you will find state-of-the-art information on: 1. Genetics and Etiology 2. Down syndrome Model 3. Neurologic, Urologic, Dental & Allergic disorders 4. Prenatal Diagnosis and Screening Whilst aimed primarily at research workers on Down syndrome, we hope that the appeal of this book will extend beyond the narrow confines of academic interest and be of interest to a wider audience, especially parents and relatives of Down syndrome patients.

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