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Biologie de l'endothélium vasculaire isolé de souris transgéniques YAC67 et YAC84- modèles murins du syndrome de Down

THÈSE dirigée par :

Dr Claudine KIEDA
Dr Danura DUŚ

Directrice de recherche – CNRS – Orléans – France
Directrice de recherche – Académie des Sciences – Wrocław -
Pologne

RAPPORTEURS :

Pr Maciej KURPISZ
Pr Irena FRYDECKA

Académie Polonaise des Sciences - Poznań - Pologne
Académie Polonaise des Sciences - Wrocław - Pologne

JURY :

Pr Wojciech GORCZYCA
Dr Claudine KIEDA
Dr Danuta DUŚ
Zofia BŁACH-OLSZEWSKA
Dr Janusz BORATYŃSKI
Pr Andrzej GAMIAN
Pr Leon STRZĄDAŁA
Pr Andrzej MAZUR
Pr Maciej UGORSKI

Académie Polonaise des Sciences – Pologne - Président du jury
CNRS - Centre de biophysique moléculaire – Orléans-France
Académie Polonaise des Sciences – Pologne
Académie Polonaise des Sciences – Pologne
Académie Polonaise des Sciences – Pologne
Académie Polonaise des Sciences – Pologne
Académie Polonaise des Sciences – Pologne
Unité de Nutrition Humaine UMR 1019, INRA, France
Académie Polonaise des Sciences – Pologne

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LIST OF ABBREVIATIONS

ACE	Angiotensin converting enzyme
AD	Alzheimer disease
AMKL	Acute megakaryoblastic leukemia
AML	Acute myeloid leukemia
Ang-1	Angiopoietin-1
aRNA	Amplified RNA
ATCC	American Type Culture Collection
BBB	Blood-brain barrier
bFGF	Basic fibroblast growth factor
BM	Bone marrow
BSA	Bovine serum albumin
CAMs	Cell Adhesion Molecules
cDNA	Complementary DNA
CNS	Central nervous system
c-PBS	Complete phosphate buffered saline
CRD	Carbohydrate binding domain
Cy	Cyanine
DMSO	Dimethyl sulfoxide
DS	Down syndrome
DSCAM	Down syndrome cell adhesion molecule
DSCR	Down syndrome critical region
DSCR-1	Down syndrome candidate region-1
EC	Endothelial cell
ECM	Extracellular matrix
EDTA	Ethylene-diamine-tetra-acetic acid
EGF	Epidermal growth factor
EHS	Engelbreth-Holm-Swarm
EPC	Endothelial progenitor cells
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
Flt1	Vegf receptor 1
Flk1	Vegf receptor 2
Flt4	Vegf receptor 3
hr	hour/hours
FTC	Fluorescein thiocarbamyl
GIRK2	G-protein inwardly rectifying potassium channel 2
GlyCAM-1	Glycosylation dependent cell adhesion molecule-1
HEV	High endothelial venules
HIF	Hypoxia-inducible factor
HSA21	Human chromosome 21
ICAM	Intercellular cell adhesion molecule (human)
Icam	Intercellular cell adhesion molecule (murine)
IFN- γ	Interferon- γ
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor-1
IgSF	Immunoglobulin superfamily
IL	Interleukin

LFA-1	Lymphocyte function-associated antigen-1
MAC-1	Macrophage-1 antigen
MAdCAM-1	Mucosal addressin cell adhesion molecule-1 (human)
Madcam-1	Mucosal addressin cell adhesion molecule-1 (murine)
MDS	Myelodysplastic syndrome
MMU10	Mouse chromosome 10
MMU16	Mouse chromosome 16
MMU17	Mouse chromosome 17
MLN	Mesenteric lymph node
NCAM	Neural cell adhesion molecule
NK	Natural killer
NO	Nitric oxide
PBS	Phosphate buffered saline
PCLP	Podocalyxin-like protein
PDGF	Platelet-derived growth factor
PE	Phycoerythrin
PECAM-1	Platelet-endothelial cell adhesion molecule-1 (human)
Pecam-1	Platelet-endothelial cell adhesion molecule-1 (murine)
PIGF	Placenta growth factor
PLN	Peripheral lymph node
PP	Peyer's patches
PSGL-1	P-selectin glycoprotein ligand-1
RT	Room temperature
SDS	Sodium dodecyl sulfate
SigLec-1	Sialic acid Ig superfamily lectin molecule-1
sLe ^x	Sialylated Lewis X
SPF	Specific Pathogen Free
SSC	Sodium citrate
TGF- β	Transforming growth factor-beta
TNF- α	Tumor necrosis factor-alpha
VCAM-1	Vascular cell adhesion molecule-1 (human)
Vcam-1	Vascular cell adhesion molecule-1 (murine)
VEGF	Vascular endothelial growth factor (human)
Vegf	Vascular endothelial growth factor (murine)
VEGFR	Vascular endothelial growth factor receptor (human)
Vegfr	Vascular endothelial growth factor receptor (murine)
VLA	Very late antigen
vWf	von Willebrand factor
YAC	Yeast artificial chromosome

PREFACE

Among the various pathological features of the Down syndrome (DS) the aberration of the immune response participates largely to the early death of the patients. This results from infections or development of autoimmunity, caused by strongly abrogated early activation, proliferation and maturation of T- and B-lymphocytes.

It was hypothesized that altered numbers of T lymphocytes can be the result of a disturbed selection of thymocytes in DS thymus, resulting in disproportions of mature T cells in the circulation. This hypothesis was consistent with the pattern of T lymphocyte expansion in people with thymic hypoplasia due to chromosome 22q11.2 deletion (Piliero, Sanford et al. 2004). Additionally, DS pathology is characterized by 10-20 fold increased risk of early development of leukemia (mostly AML) (Hitzler and Zipursky 2005).

A murine model of DS, presented by transgenic mice with an additional copy of human *GIRK2* gene was created. These mice display, besides mental retardation and heart failures, depressed immune response due to disabled immune system and altered distribution of T lymphocyte populations. The latter is attributed to changes in the proportions among mature T lymphocytes subpopulations, characterized by higher level of CD4⁺TCRαβ⁺ cells, modifications in the number of CD25⁺ cells, as well as changed ratio of CD4⁺CD8⁺ / CD4⁺CD8⁻ cells as compared to the control FVB mice.

The cause of such changes was looked for, taking into account a thymic disfunction possibility. Because differences did not seem to appear during lymphocytes maturation in the thymus (Bichler 2002), we hypothesized that the modification could result either from an abnormal exportation of the matured T lymphocytes from the thymus, or from the differences in the distribution of those cells among the lymphoid organs, which is regulated and controlled by lymphocytes homing/recirculation processes.

Both phenomena: thymic exportation and homing/recirculation of lymphocytes, in the various organs and tissues, are under the control of endothelia. The endothelial cells that line the vessels are establishing the barrier that regulates cell trafficking. Consequently, they control the proportions of the various lymphocyte subpopulations residing in tissues or circulating in the blood.

We hypothesize that the endothelial cells might play an important role in the differential distribution of lymphocytes in the DS pathology. Consequently, we designed an *in vitro* EC cell lines model to study ECs from the transgenic mice, as compared to their

normal counterparts, in term of their organospecificity status, and adhesion, recognition and selection abilities toward lymphoid cells.

1. INTRODUCTION

1.1 DOWN SYNDROME GENERALITIES

Down syndrome (also Down's syndrome; DS) or trisomy 21 is a chromosomal disorder resulting from the presence of all or part of an extra 21st chromosome. Trisomy 21 is the most common human autosomal aneuploidy compatible with postnatal survival. It occurs in 1 out of 700 live births in all ethnic groups (Reeves, Baxter et al. 2001).

There is a pronounced maternal-age effect on the occurrence of trisomy 21, with increased risk when maternal age passes 35 years (Reeves, Baxter et al. 2001). Among women under the age of 25 years about 2% of all clinically recognized pregnancies are trisomic, when in the case of women over 40 years this value approaches 35% (Hassold and Chiu 1985).

Down syndrome is named after John Langdon Down, the British doctor who first described it in 1866. He grouped people with Down syndrome as having features of Mongolian people what established the name of this discrepancy as mongolism (Down 1866). In 1965 World Health Organization decided to change this name as an inappropriate into Down syndrome (Patterson and Costa 2005).

In 1959 Jérôme Lejeune discovered that this autosome disorder is caused by triplication of chromosome 21st (Lejeune, Gautier et al. 1959; Lejeune and Turpin 1962).

Three types of mutations causing the Down syndrome can be distinguished:

- Full trisomy 21 (95%) – the most common, when entire chromosome 21 is present in all cells of the body, usually resulting from nondisjunction in meiosis, most frequently in female meiosis I (Lamb, Feingold et al. 1997; Antonarakis, Lyle et al. 2004).
- Mosaicism (1%) – additional chromosome is present in some cells of the body. Mosaicism can occur when the initial zygote with trisomy 21 lost one 21st chromosome because of mitotic (somatic) error or when the initial zygote had two 21st chromosomes, but in the course of cell division one of the 21st chromosomes was duplicated (Pangalos, Avramopoulos et al. 1994).
- Partial trisomies (4%) – they result from abnormal meiosis and segregation of chromosomes. In this type of mutation Robertsonian and non-Robertsonian chromosome translocations can be distinguished (Antonarakis, Lyle et al. 2004).

1.2 DOWN SYNDROME FEATURES

Additional copy of the chromosome 21 may result in an increased level of transcripts and proteins in a 1.5- to 3-fold order (Reeves, Baxter et al. 2001) or in the repression of several transcripts (Epstein 1990). Additionally, triplicated genes can interact with each other and affect expression of genes located on other chromosomes. This can affect many aspects of organism development, producing a wide and variable set of clinical features in a given individual (Reeves, Baxter et al. 2001). There are many physical features characteristic for DS persons, some of these also appear in people with a standard set of chromosomes (Villar 2005).

1.2.1 Down syndrome disabilities

Trisomy 21 is not a causative factor but increases the predisposition for some diseases and alterations. Table 1 shows the percentage or increased occurrence of the most common Down syndrome associated pathologic features.

The mental retardation is characteristic for all persons with DS but its intensity varies greatly from one individual to another. Nowadays the exact cause of it is still unknown. Researchers have listed the errors during the brain development in the fetus, atrophy of cells in the frontal area of the brain and dysfunction of the individual brain cells. Although none of the genes on chromosome 21 can as yet be related to these structural defects, genes expressed in the developing and adult brain are good candidates. These include *DSCAM* gene (Down syndrome cell adhesion molecule) (Yamakawa, Huot et al. 1998). Within the brain, *DSCAM* is expressed in the cerebral cortex, hippocampus and cerebellum (Barlow, Micales et al. 2001), all of which are altered in DS (Jernigan, Bellugi et al. 1993; Golden and Hyman 1994; Raz, Torres et al. 1995). Recent research has focused on the *DYRK* gene, located on the 21st chromosome. This gene is very active in the fetal brain, and disruption of this gene in trisomic mice has been found to cause learning difficulties (Cohen W.I. 2002).

Muscle hypotonia is a feature common to almost all children with DS. It can be the cause of delayed gross motor development (such as crawling and walking), constipation and gastroesophageal reflux (Cohen 2002).

Heart disease and defects of the cardiovascular system are responsible for the majority of premature deaths caused by congenital defects (Cohen W.I. 2002). About half of children with Down syndrome are born with congenital heart disease (Freeman, Taft et al. 1998).

Phenotype	% in DS or fold increase in DS
Mental retardation	100%
Alzheimer disease neuropathology	100% after 35 years
Muscle hypotonia	100%
Characteristic dermatoglyphics	85%
Short stature	70%
Brachycephaly	75%
Epicanthic folds	60%
Iris Brushfield spots	55%
Protruding tongue	45%
Folded/dysplastic ear	50%
Short, broad hands	65%
Short fifth finger	60%
Congenital heart defect	40%
Atrioventricular canal	16%
Duodenal stenosis/atresia	250x
Imperforate anus	50x
Hirschsprung disease	30x
Acute megakaryocytic leukemia	200-400x
Leukaemia (both ALL and AML)	10-20x

Table 1. Frequency of Down syndrome (DS) phenotypes (Antonarakis, Lyle et al. 2004)

It is estimated that 7-10% of children with DS suffer from autistic-like behavior. These children have more problems with speech and learning. Closely connected with Down syndrome is Alzheimer disease (AD). The onset of AD in adults with DS is known to be 20 or more years earlier than in aging adults from the general population. By the age of 40 years, almost all adults with DS have characteristic features in the brain that are associated with AD (including deposition of amyloid- β protein in diffuse and neuritic plaques in brain) but only 20-25% of them show any of the dementia or cognitive decline that is the hallmark of Alzheimer disease (Dalton 1998; Dalton 1999; Park, Alberti et al. 2000; Cohen 2002). Between 38% and 78% of DS individuals exhibit some hearing loss, which is usually caused

by the chronic presence of fluid in the ears (conductive), dysfunction of the transfer of sound from the inner ear to the brain (sensorineural), or a mixture of both types (Cohen W.I. 2002).

People with DS produce less growth hormone which is one of the reasons of their short stature. Other endocrinological problem is hypothyroidism which can manifest at any age. In infants and newborns with hypothyroidism the most common cause of this disease is a failure of the thyroid gland to develop correctly. In older children and adults, the most common cause is an autoimmune reaction against thyroid tissue. The symptoms of hypothyroidism can be: decreased growth rate, weight gain, constipation, lethargy, decreased muscle tone and dry skin (Cohen W.I. 2002).

1.2.2 Immunological status in Down syndrome

During the last 2 decades, the median life length of people with DS has doubled and presently reaches about 50 years. Nevertheless, the mortality in DS is still higher than in the general population. Many publications have reported abnormalities of the immune system in Down syndrome persons. They suffer mostly because of gastrointestinal as well as respiratory infections, which are the major cause of death in this group (Glasson, Sullivan et al. 2002; Yang, Rasmussen et al. 2002). As an example, the mortality in case of sepsis can be mentioned (Garrison, Jeffries et al. 2005).

Various factors were proposed to explain above mentioned disturbances in DS persons, but available data describing the changes in immune system are inconsistent. They can be influenced by peoples age, race and different demographic factors, including geographic site, and others (Douglas 2005). Also age-related variations in lymphocyte subpopulation sizes, due to the expansion and maturation of the immune system in the first years of life (Comans-Bitter, de Groot et al. 1997; de Vries, de Groot et al. 1999) cause differences among the various studied populations and between DS group and controls (de Hingh, van der Vossen et al. 2005). Nevertheless, various observations and immunological theories to explain these differences have been published. Consequently, few facts are known to date:

One of the abnormalities found in newborns with DS is low platelet count, (thrombocytopenia). Sometimes, the platelet count may be so low that transfusion of platelets may be necessary to prevent bleeding problems. The reason for the thrombocytopenia is unknown (Cohen W.I. 2002).

It is also known that children with DS have strongly abrogated early activation, proliferation and maturation of T and B lymphocytes in the first year of life, what normally takes place in response to continuous encounters with environment antigens (de Hingh, van der Vossen et al. 2005). This abrogation of early expansion of the lymphocytes is irrespective of the frequency of infections or development of autoimmunity. Altered proportions of lymphocytes was supposed to be caused by increased deletion of thymocytes in the Down syndrome thymus resulting in a diminished proportion of mature T cells (Douglas 2005). It has been also hypothesized that the decreased number of T cells could be explained by thymic alterations characteristic for DS (Ugazio, Maccario et al. 1990; Murphy, Insoft et al. 1995). Consistent with this hypothesis is the fact that a similar pattern of T lymphocyte expansion in the first years of life is also seen in people with thymic hypoplasia due to chromosome 22q11.2 deletion (DiGeorge syndrome) (Jawad, McDonald-McGinn et al. 2001).

Among the immunological changes which have been also reported in Down syndrome was a low number of circulating B cells, which remained severely decreased in children as well as in adults (Lockitch, Singh et al. 1987; Cossarizza, Ortolani et al. 1991). Perturbed proportions in T cell subpopulations was also observed, resulting in an inverted CD4/CD8 ratio (decreased CD4⁺ counts vs increase in CD8⁺ counts) (Bertotto, Gerli et al. 1984; Karttunen, Nurmi et al. 1984; Lockitch, Singh et al. 1987; Cuadrado and Barrena 1996). Another phenomenon is the increased proportion of lymphocytes highly expressing LFA-1 (*lymphocyte function-associated antigen-1*) whereas in non-trisomic organisms, low expressing LFA-1 lymphocytes predominate (Barrena, Echaniz et al. 1992). Also an increase of the peripheral T cells expressing TCR- γ/δ (Murphy and Epstein 1992; Cuadrado and Barrena 1996) and activated T cells (CD3/HLA-DR-positive cells) (Licastro, Mariani et al. 2001) have been reported. Furthermore, Down syndrome population of lymphocytes is also characterized by increased numbers of cells with NK (natural killer) cells activity, such as CD16, CD56 and CD57 (Cossarizza, Ortolani et al. 1991).

Down syndrome pathology is characterized by 10-20 fold increased risk of leukemia (Fong and Brodeur 1987; Avet-Loiseau, Mechinaud et al. 1995). At the same time solid tumors of childhood and adult non-hematological cancers are significantly less frequent, with the complete absence of breast cancer in persons with DS, than in age-matched controls. Moreover, no leukemia was observed in persons aged over 29 (Hasle, Clemmensen et al. 2000).

The most unique feature of DS children is transient leukemia which appears in 10% of newborn infants with DS (Zipursky 2003). Those children have clonal population

of megakarioblasts in their peripheral blood (Kurahashi, Hara et al. 1991; Miyashita, Asada et al. 1991). In most cases these cells disappear spontaneously during the first 3 months of life, but approximately 20% of children can develop myelodysplastic syndrome (MDS) or acute megakaryoblastic leukemia (AMKL) in the first 4 years of life (Tchernia, Lejeune et al. 1996; Zipursky 2003; Hitzler and Zipursky 2005).

1.3 GENETICS OF DOWN SYNDROME

Down syndrome is a chromosomal disorder caused by the presence of all or part of an extra 21st chromosome. As it was already mentioned it was assumed that this additional copy results in increased levels of transcript and protein, on the order of 1.5- to 3-folds (Reeves, Baxter et al. 2001). Not all DS features are present in every individual with trisomy 21. Moreover the severity of those features which are present can vary (Epstein, Korenberg et al. 1991). This variability could be caused by different allele combinations of genes located not only on chromosome 21 but also on other chromosomes, as well as the genetic background of the individual in whom the trisomy 21 occurs as well as individual sensitivity to environmental influences (Reeves, Baxter et al. 2001).

There are two hypothesis explaining how additional chromosome 21 presence could result in disruption of normal patterns of development: ‘developmental instability’ hypothesis and ‘gene-dosage effect’ hypothesis. According to the first hypothesis the correct balance of the expression of genes regulating development is disrupted by dosage imbalance of the hundreds of genes located on chromosome 21 (Reeves, Baxter et al. 2001). This hypothesis goes along with the fact that features seen in DS also occur in other trisomies and the individuals with trisomy 21 display a high variability of the DS features. The second, ‘gene-dosage’ hypothesis, says that the DS features are caused by imbalance of a specific individual gene or group of genes from chromosome 21. It was created on a basis of observations of DS individuals with partial trisomy of chromosome 21. DS persons with minimally overlapping segments of chromosome 21, which have shown the same specific DS feature, let to create phenotype maps of the chromosome (Reeves, Baxter et al. 2001). However this map may not be precise due to the fact that there is no human beings having just minimal overlapping region which could be assigned to the given phenotype (Reeves, Baxter et al. 2001).

Investigating the individuals with partial trisomy 21 allowed to identify the region on chromosome 21 which is thought to be responsible for most of the DS features (Korenberg, Chen et al. 1994; Rachidi and Lopes 2007). It is called Down syndrome critical region (DSCR) and is located on a distal part of a long arm. The most precise description of the DSCR says of about 5.4 Mb on human chromosome 21 from a proximal boundary between markers *D21S17* and *D21S55* to a distal boundary between *MX1* and *BCEI* (Olson, Roper et al. 2004; Olson, Roper et al. 2007). However, recently the concept and existence of the DSCR has been challenged. Using the transgenic mice Ts1Rhr (described below) Olson *et al* have shown that trisomy for the DSCR alone is not sufficient to produce the structural and functional features of brain impairment that are seen in the Ts65Dn mouse and DS (Olson, Roper et al. 2007).

Human chromosome 21 (HSA21) is the smallest human autosome. In May 2000, the DNA sequence of the long arm of it and a catalogue of the genes on the chromosome was published. The gene content of human chromosome 21 (HSA21) has been estimated of 225 genes, 127 correspond to known genes and 98 represent novel genes (Hattori, Fujiyama et al. 2000).

Since the sequence of the chromosome 21 was published, analysis of its content continues, and the precise number of genes is changing according to the categorization of genes as more research is carried out. Nevertheless the functions of most of these genes remain still largely unknown. Even knowing the molecular defect, it is difficult to decipher the complex pathophysiology of the disease, the developmental consequences of the trisomy and the impact on behavior and cognitive function. Due to the fact that some genes are highly regulated, the impact of gene overdosage on the transcript level may vary. Henceforth the DNA sequence of chromosome 21, as well as the complete human genome sequence, may allow for more direct and comprehensive approach to understand Down syndrome (Cohen W.I. 2002).

1.4 MURINE MODELS FOR DOWN SYNDROME

It is generally accepted that in DS chromosome 21 transcripts are overexpressed by about 50%. However, this assumption is difficult to test in humans due to limited access to tissues. No single mechanism can explain the harmful consequence of trisomy 21, and therefore, there is no simple solution to counteract its phenotypic impact.

It is known that HSA21 has regions of conserved synteny to mouse chromosome 16 (MMU16) (Fig. 1.1). Therefore, mice trisomic for chromosome 16 have been generated and investigated as a mouse model for Down syndrome. However, mice with additional entire chromosome 16 (Ts16) never survive the first day of birth and most frequently die during late gestation (Kola and Hertzog 1997; Hattori, Fujiyama et al. 2000; Mural, Adams et al. 2002; Guigo, Dermitzakis et al. 2003). However, during embryonic development these mice show anatomic features that are reminiscent of Down syndrome (Lacey-Casem and Oster-Granite 1994), and newborn animals have significant morphological, biochemical and immunological similarities to DS individuals (Kola and Hertzog 1997; Hattori, Fujiyama et al. 2000; Mural, Adams et al. 2002; Guigo, Dermitzakis et al. 2003). Therefore, the use of mouse model is still one of the most promising approaches to understand DS pathology.

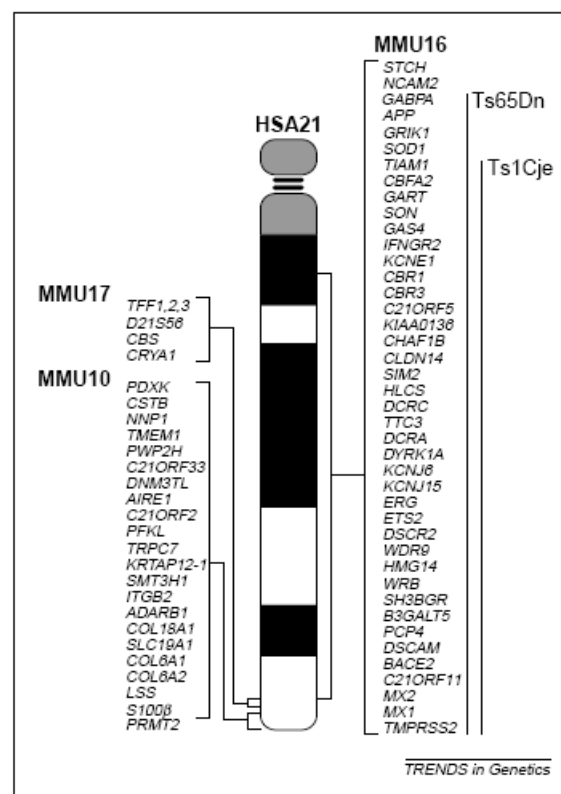


Figure. 1.1. Comparative genetic maps of human chromosome 21 (HSA21) and mouse chromosomes 16 (MMU16), 17 (MMU17) and 10 (MMU10) (Ts65Dn and Ts1Cje – transgenic mouse models for Down syndrome, details in text) (Reeves, Baxter et al. 2001)

Nevertheless, this model presents some disadvantages, like the fact that part of the genes with homology to HSA21 is also located on murine chromosomes 3 (MMU3), 10 (MMU10) and 17 (MMU17). Moreover, MMU16 includes genes that are not present on HSA21. Consequently, trisomy of chromosome 16 does not entirely mimic Down syndrome.

To overcome the problem of early lethality, investigators have created mice that are trisomic for a part of MMU16 (Fig 1.2) (Davisson, Schmidt et al. 1990; Sago, Carlson et al. 2000).

The most widely used is Ts65Dn mouse. The trisomy in this mouse extends for at least 23.3 Mb: from *Mrpl39* to the *Znf295* genes (Kahlem, Sultan et al. 2004) and contains approximately 132 genes that are homologous to HSA21 (Antonarakis, Lyle et al. 2004). The Ts65Dn mice display a variety of phenotypic abnormalities including early developmental delay, reduced birth weight, muscular trembling, male sterility, abnormal facies, age-related degeneration of basal forebrain cholinergic regions, learning and behavioural deficits (Reeves, Irving et al. 1995; Antonarakis 1998).

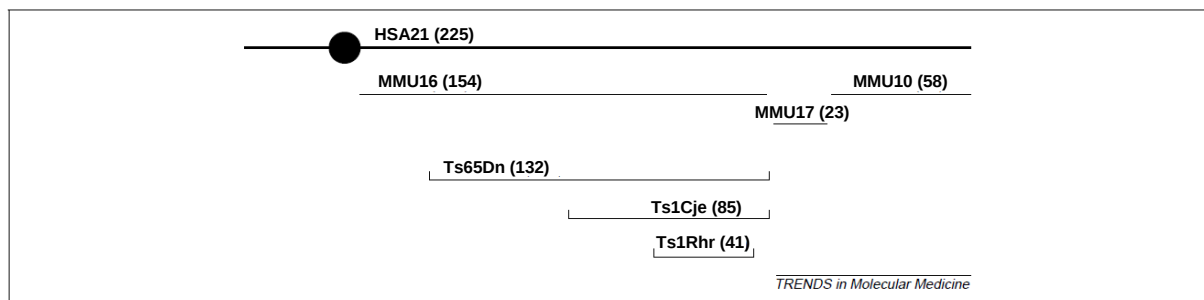


Figure 1.2. Gene content of HSA21 and orthologous regions of MMU16, MMU17 and MMU10; gene numbers were adapted from (Antonarakis, Lyle et al. 2004)
HSA21 - human chromosome 21; MMU16, 17, 10 - mouse chromosomes 16, 17, 10

The Ts1Cje mouse is trisomic for the segment spanning from the *Sod1* to *Znf295* gene. Ts1Cje mice contain about 85 genes with HSA21 orthologues (Antonarakis, Lyle et al. 2004). Ts1Cje mice are fertile, show milder behavioral aberrations than Ts65Dn and have no noticeable degeneration of basal forebrain cholinergic neurons (Sago, Carlson et al. 1998). Ts1Cje carry only the subset of the triplicated genes found in Ts65Dn, nevertheless several DS phenotypic features such as craniofacial abnormalities (Richtsmeier, Zumwalt et al. 2002) and reduced cerebellar volumes (Olson, Roper et al. 2004) are observed in both DS models. Additionally Ts1Cje mice exhibit abnormalities in hippocampal synaptic plasticity, long-term

potentiation and long-term depression, but these are less marked than in Ts65Dn mice (Siarey, Villar et al. 2005).

The next mouse model for Down syndrome is Ts1Rhr mouse with a trisomy of the subset of genes corresponding to the DSCR. It was believed that Ts1Rhr could directly determine whether trisomy for the DSCR is sufficient to produce phenotypes seen in Ts65Dn and DS and hence estimate the role of DSCR in DS pathology. However, analysis of Ts1Rhr model has demonstrated that trisomy for the DSCR alone is insufficient and not necessary to cause such features attributed to Down syndrome, as short stature, small mandible (which contributes to the protruding tongue) and anomalies of the craniofacial skeleton (Olson, Roper et al. 2004; Olson, Roper et al. 2007).

All above listed approaches do not provide a model identifying the individual function of the genes and their role in DS, but the overexpression of a single gene or a small group of genes may help to identify genes related to specific pathophysiological features (Dierssen, Fillat et al. 2001).

1.5 TRANSGENIC MICE YAC67 AND YAC84

Transgenic mouse *in vivo* library has been developed by inserting yeast artificial chromosomes (YACs) bearing a fragment of human chromosome 21 into the murine genome. There is a controversial evidence that this region may be particularly important in the pathogenesis of the syndrome (Rahmani, Blouin et al. 1989).

DS mouse models which allow to study DSCR role are mice created by Smith *et al.* (Smith, Zhu et al. 1995). They selected four chromosomal fragments, containing 1 to 10 genes of HSA 21 which covered the region *D21S17-ETS2*. Most of these genes were found on the syntenic region of MMU 16. These four fragments were incorporated into yeast artificial chromosomes giving as follows: YAC230E8, YAC141G6, YAC152F7 and YAC 285E6 (Fig. 1.3). First three above mentioned YACs contained 10, 4 and 4 genes respectively. Next, each YAC was incorporated into a mouse genome inducing partial trisomy for the genes that it carried. The fourth heterozygous mice contained human *GIRK2* gene (*KCNJ6*) in one (YAC67) or more (YAC84) copies (Smith, Zhu et al. 1995). Homozygous animals were selected by Lignon *et al.* (Lignon, Bichler et al. 2008). They contain two (YAC67) or more (YAC84) additional *GIRK2* copies (Roubertoux, Bichler et al. 2005) (Lignon personal communication).

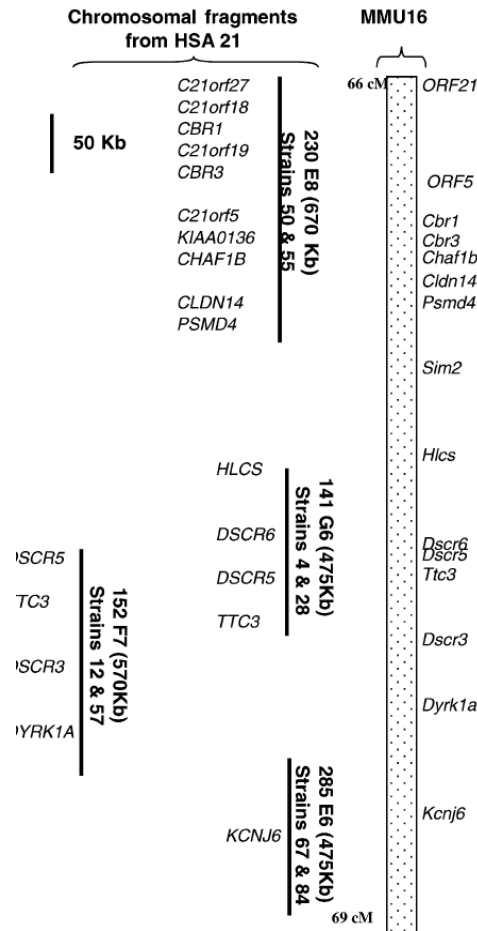


Figure 1.3. YAC 285E6, YAC141G6, YAC230E8 and YAC152F7 incorporating HSA 21 fragments and corresponding regions on MMU16
HSA 21 – human chromosome 21; MMU16 – mouse chromosome 16, *Kcnj6* – *Girk2*

One of the best known genes located on chromosome 21 is *GIRK2* (known also as BIR1, hiGIRK2, inwardly rectifying K^+ channel Kir3.2, KATP2, KATP-2, KCNJ6, KCNJ7, KIR3.2). *GIRK2* is expressed mainly in brain. Here its expression is modulated according to the localization. Being a member of potassium channel proteins, GIRK2 is responsible for influx of potassium ions, conducting an outward hyperpolarizing current and maintaining resting membrane potential (Dascal 1997; Faraci and Heistad 1998). GIRK2 channels can mediate specific reactions, including anxiety and convulsions (Blednov, Stoffel et al. 2001). This channel could be also involved in the onset of cell migration (Chen, Ehrhard et al. 1997; Lauritzen, De Weille et al. 1997).

Generally, GIRK channels are thought to be heteromultimers consisting of 2-4 distinct *GIRK* genes products, such as GIRK1₂/GIRK4₂ (Silverman, Lester et al. 1996), GIRK2/GIRK3 (Jelacic, Kennedy et al. 2000), or isoforms of one gene e. g. GIRK2a/GIRK2c (Inanobe, Horio et al. 1999).

GIRK2 channel is activated by neuromodulators acting on: m₂ muscarinic, α₂ adrenergic, D₂ dopaminergic, histamine, serotonin 5HT_{1A}, A₁ adenosine, γ-aminobutyric acid_B, μ-, κ-, and δ-opioid, as well as somatostatin receptors (Inanobe, Yoshimoto et al. 1999; Mark and Herlitze 2000; Blednov, Stoffel et al. 2003). The activation is mediated by direct binding of G protein subunit G_{βγ} to the GIRK channel (Krapivinsky, Krapivinsky et al. 1995; Yamada, Inanobe et al. 1998; Mark and Herlitze 2000).

Several mouse *Girk2* splice variants have been described: GIRK2A, GIRK2B, GIRK2C and GIRK2D with different amino acid residues numbers in the C-terminal end of the molecule. The longest one is GIRK2C (Isomoto, Kondo et al. 1996; Wei, Hodes et al. 1998; Inanobe, Horio et al. 1999). The isoforms of GIRK2 gene are differentially expressed within tissues. While GIRK2A was detected in forebrain, cerebellum and eyes, the GIRK2B was distributed ubiquitously (Isomoto, Kondo et al. 1996). GIRK2C and GIRK2D are characteristic for brain, mostly for cerebellum (Wei, Hodes et al. 1998).

In the present work, homozygous transgenic mice with inserted YAC 285E6 (yeast artificial chromosome carrying only one human *GIRK2* gene) were investigated.

Transgenic mice containing an additional copy of human *GIRK2* gene in the YAC 285E6 performed normally on Morris water maze (Smith, Stevens et al. 1997) but demonstrated mental retardation, heart failure as well as a disabled immune system as compared to their non-transgenic counterparts (Bichler 2002; Chabert, Jamon et al. 2004; Lignon, Bichler et al. 2008). The last has been attributed to changes in the proportions among T lymphocytes populations, characterized by changed ratio of CD4⁺CD8⁺ / CD4⁺CD8⁻ cells and higher level of CD4⁺TCRαβ⁺ cells, as well as modifications in the number of CD25⁺ cells. However those differences did not appear to be the result of the impaired lymphocytes maturation in the thymus (Zoë Bichler, Danielle Migliore-Samour, personal communication).

In the newborn organism, the primary lymphoid organs i.e. bone marrow and thymus, are responsible for the production of functionally mature naive lymphocytes from non-functional progenitors. Secondary lymphoid organs: lymph nodes, gut-associated lymphoid organs (Peyer's patches) spleen and appendix are the meeting points between antigens and lymphocytes. They are specialized in the accumulation and presentation of antigens to the naive and memory lymphocytes and thus represent the sites of secondary lymphoid

differentiation. Lymphocytes exported from the primary lymphoid organs have to home in a selective and strictly controlled manner into the secondary lymphoid organs in their search for the antigens they should respond to, this process is called homing. They also recirculate *via* the blood and lymphatic circulation from one lymphoid organ to another. Those movements are controlled by the portal constituted by the endothelium (Janeway 2005).

Therefore, it was hypothesized that the altered T lymphocytes proportions observed in the transgenic mice could emerge from the changes in biology of endothelial cells, which may result in disturbed T lymphocytes-endothelium interactions, first at the level of the mature T cells exportation from the thymus and then at the level of the other lymphoid organs entry and exit, thus resulting in the modification of the proper proportions of circulating T cells.

1.6 ENDOTHELIAL CELLS

1.6.1. Generalities on the biology of endothelial cells

Endothelial cells (ECs) form a continuous monolayer between blood and surrounding tissues. The surface of the endothelium in an adult human is composed of approximately $1-6 \times 10^{13}$ cells and covers an area of approximately $1-7 \text{ m}^2$ (Ribatti, Nico et al. 2002). Endothelia are selective filters which regulate the passage of gases, fluids, molecules and cells. The transfer of fluids and molecules through endothelial barrier is executed by endocytosis, transcytosis, intracellular transport *via* caveolae and channels, or goes paracellularly, *via* intercellular junctions (Simionescu, Gafencu et al. 2002). The molecules engaged in the process are membrane-bound receptors for numerous molecules, such as growth factors, coagulant and anticoagulant proteins; lipid transforming proteins, metabolites (e.g., nitrous oxide and serotonin) and hormones. ECs carry also specific junction proteins and adhesive receptors that govern cell-cell and cell-matrix interactions (Ribatti, Nico et al. 2002).

Endothelium plays a crucial role in vascular tone, inflammation, innate immunity, prothrombosis/coagulation and fibrinolysis. ECs are directly involved in the development and progression of stroke, peripheral vascular disease, venous thrombosis, insulin resistance, tumour growth and metastasis, and adverse reproductive outcomes. Hence, endothelium is a major factor in the development of vascular diseases in general and of atherosclerosis in particular (Hooper, Catravas et al. 2007).

The common markers of ECs are von Willebrand factor (vWf) and angiotensin converting enzyme (ACE). Von Willebrand factor is a glycoprotein involved in hemostasis.

It is stored in Weibel-Palade bodies and its release from these granules occurs in a regulated manner (Vischer and de Moerloose 1999). It plays a key role in primary homeostasis, as the adhesion promotor of platelets to the vascular endothelium, but also functions as a plasma-carrier protein for coagulation factor VIII (FVIII) (Nichols, Samama et al. 1995; Vischer and de Moerloose 1999). It is expressed at higher levels on the venous than on the arterial sites of the capillary circulation (Page, Rose et al. 1992). Another molecule considered as an EC marker is ACE, a large glycoprotein spanning the cell membrane. ACE regulates the blood pressure and fluid homeostasis through inactivation of the vasodilator bradykinin, by the sequential removal of its two C-terminal dipeptides (Dzau, Bernstein et al. 2002). Additional markers considered as typical for ECs are CD31 and VE cadherin (Paprocka 2000).

Endothelial cells are a heterogeneous population. There are differences in ECs phenotypes from large and small vessels, between cells in different activation state, as well as between organ and tissues, which is called organoselectivity (Springer 1994; Kieda, Paprocka et al. 2002). Both blood and lymphatic vessels are lined with ECs but the latter may be distinguished by the expression of some specific markers like podoplanin or LYVE-1 (Oliver and Detmar 2002).

Endothelium layer can be either thick (more than 2 μ m) in skeletal tissue, cardiac smooth muscle, testes and ovary tissue or thin (less than 1 μ m) in central nervous system and dermis (Ribatti 2006). The most specialized endothelium, found in secondary lymphoid organs, seemed to be HEV (*high endothelial venules*), participating in physiological lymphocyte circulation. HEV is characterized by the expression of addressins which direct specific lymphocyte homing. These are GlyCAM-1 (*glycosylation dependent cell adhesion molecule-1*) and CD34 in peripheral lymph nodes and MAdCAM-1 (*mucosal addressin cell adhesion molecule-1*) in Peyer's patches, mesenteric lymph nodes and lamina propria of the gut. In contrast to flat morphology of ECs from other vessels, HEV cells have plump, cuboid appearance. In HEV cells are connected by discontinuous, spot-welded junctions, which facilitate lymphocyte passage and extravasation. In case of chronic inflammation HEV-like structures may be also found in different extralymphoid sites (Kieda, Paprocka et al. 2002; Kieda 2003; Janeway 2005).

On the contrary, the tightest endothelial layer in the body is present in the central nervous system (CNS). In brain it constitutes the blood-brain barrier (BBB) which is an effective blockade to fluid and molecule transfer. Unlike the endothelium from the rest of the body, there is no fenestrations in BBB, and the ECs are joined together by tight

junctions which under normal physiological conditions help to limit the paracellular flux of hydrophilic molecules, with the exception of the smallest, lipophilic molecules (Ballabh, Braun et al. 2004). In the brain the tight junctions are so well developed that even ionic permeability is severely limited (Staddon and Rubin 1996). The endothelial cells of the BBB contain various membrane transporters which participate in the influx and efflux of various substrates such as glucose, electrolytes, nucleosides and amino acids. Membrane permeation involves passive diffusion, carrier-mediated (facilitative), and/or ATP-dependent (active) processes (Lee, Dallas et al. 2001).

1.6.2 Angiogenesis

Angiogenesis is the process of formation of vessels in post-embryonic development, reproduction and wound healing (Folkman and Shing 1992). It is also of importance in many pathological situations, such as tumor growth and metastasis, rheumatoid arthritis, proliferative diabetic retinopathy or atherosclerosis (Chavakis and Dimmeler 2002). Angiogenesis process involves several steps.

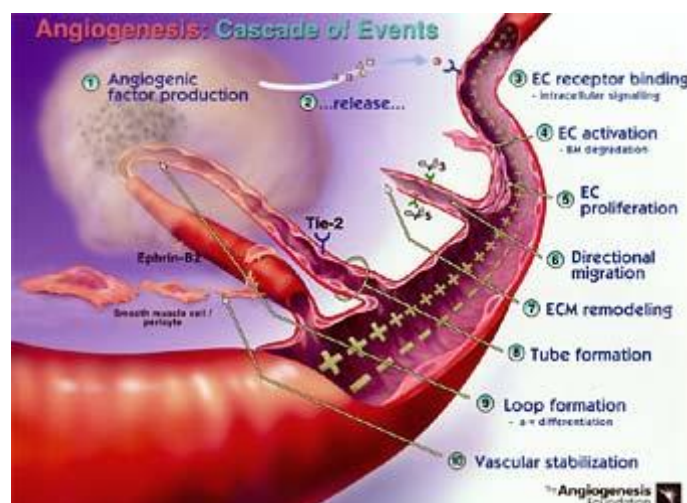


Figure 1. 4. The process of angiogenesis

Tissue can stimulate the process of angiogenesis by production pro-angiogenic stimuli (1). This stimulus is released (2) and binds to appropriate receptors (3) localized on the surface of endothelial cells. Endothelial cells are activated (4), what causes their proliferation (5) and migration (6) towards the tissue which has produced pro-angiogenic stimulus. The enzymes like matrix metalloproteinases dissolve the tissue in front of the sprouting vessel tip in order to accommodate it (7). Cells roll up to form a blood vessel tube (8) which creates a loop in which blood can circulate (9). At the end newly formed blood vessel tubes are stabilized by muscle cells, pericytes (10) (The Angiogenesis Foundation).

Like it is visualized in Figure 1.4 stimulation is caused by proangiogenic factors binding to specific receptors. The main angiogenic factors produced in inflamed, ischemic or hypoxic tissues are VEGF (*vascular endothelial growth factor*), FGF (*fibroblast growth factor*) and Ang-1 (*angiopoietin-1*). Activated endothelial cells release proteases that degrade the basement membrane. Proteolysis is one of the earliest and most sustained activities which enable endothelial cells to be released from the original vessel walls. The endothelial cells begin to form solid sprouts which can be connected to the neighboring vessels. As sprouts extend toward the source of the angiogenic stimulus, endothelial cells migrate in that direction, using adhesion molecules. They roll up, form loops and create a blood vessel tube. New blood vessel is stabilized by accessory cells: smooth muscle cells and pericytes (Dimmeler and Zeiher 2000; Chavakis and Dimmeler 2002).

VEGFs are a family of growth factors that includes placenta growth factor (PlGF), and the various forms of VEGF i.e. VEGF-A, VEGF-B, VEGF-C, and VEGF-D (Ferrara 2004). VEGF exists either in soluble form, ECM (extracellular matrix) or membrane bound growth factor, or as component of the ECM itself (Griffioen and Molema 2000). VEGF binds to receptors with tyrosine kinase activity: Flt-1 (VEGFR-1) and Flk-1 (KDR/VEGFR-2), Flt-4 (VEGFR-3). They are expressed on vascular endothelium, on monocytes, macrophages and also on some tumor cells. Interactions of VEGF with Flk-1 (in the case of blood vascular ECs) induce a signal transduction pathway in endothelial cells through Flk-1-dimerization, resulting in permeability enhancement, cellular proliferation, and migration (Griffioen and Molema 2000)

FGF (*fibroblast growth factor*) takes part in cell migration, proliferation, and differentiation (Kanda, Hodgkin et al. 1997). The best known members of the FGF family, are aFGF (*acidic fibroblast growth factor*) and bFGF (*basic fibroblast growth factor*). The cellular effects of FGFs are mediated *via* their specific binding to high-affinity tyrosine kinase receptors (Klein, Roghani et al. 1997) (Carmeliet 2000).

Angiopoietin-1 (Ang-1) is a promoting angiogenesis glycoprotein, secreted in response to hypoxia. Its receptor, Tie2, is generally restricted to the endothelium. The interactions between Ang-1 and Tie2 are important during development, tumor growth, and wound healing (Sato, Tozawa et al. 1995; Lin, Polverini et al. 1997; Wong, Haroon et al. 1997; Stratmann, Risau et al. 1998).

In several pathological conditions angiogenesis is stimulated by **hypoxia**. This process is undertaken through HIF (*hypoxia-inducible factor*) stimulation, which influences

the expression of the genes implicated in angiogenesis, cell survival/death, pH regulation, adhesion, extracellular matrix remodeling, migration and metastasis (Brahimi-Horn, Chiche et al. 2007).

1.6.3. The role of adhesion in lymphocyte homing and recirculation

One of the key functions of endothelium is to control motility/migration and homing of lymphocytes and their recirculation through HEV. Homing is a physiological process by which lymphocytes seek out and localize into a particular tissue and microenvironment.

In human body there are 10^{12} lymphocytes and they circulate continuously from the bloodstream to the lymphoid organs and back to the blood, making contacts with antigen-presenting cells in the lymphoid tissue, using adhesion molecules (Fig. 1.5, 1.6) (Janeway 2005). In figure 1.5 lymphocytes adhering to *in vitro* cultured ECs are presented.

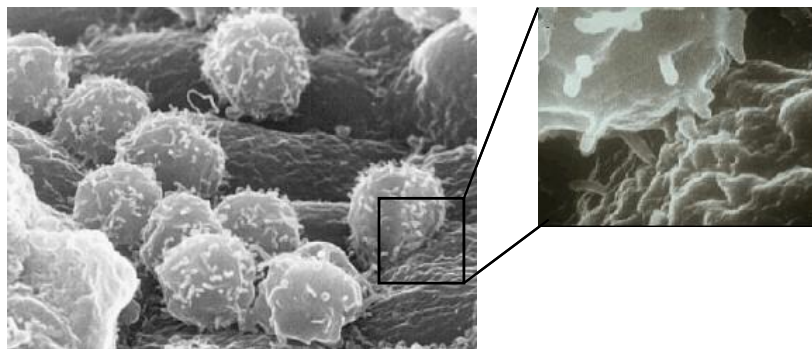


Figure 1. 5. Scanning electron microscopy of luminal surface of a rat high endothelial venule showing adherent lymphocytes (Anderson and Anderson 1976)

1.6.3.1 Cell Adhesion Molecules (CAMs)

Most of the CAMs belong to the following protein families: Ig (immunoglobulin) superfamily (IgSF CAMs), integrins, cadherins, selectins, lymphocyte homing receptors, and also mucin-like molecules. Some of these molecules are concerned mainly with lymphocyte migration and homing, others have broader roles in the generation of immune response (Butcher 1992; Butcher and Picker 1996; Aplin, Howe et al. 1998).

Selectins (CD62) belong to a family of adhesion molecules classified as C-type lectins, due to a calcium-dependent carbohydrate binding domain (CRD) specific for sialylated sugar moieties. There are three selectins: E-selectin (CD62E; in endothelial cells), P-selectin (CD62P; in platelets and endothelial cells) and L-selectin (CD62L; in leukocytes). The best-characterized ligand for the three selectins is PSGL-1 (*P-selectin glycoprotein ligand-1*), which is a mucin-type glycoprotein expressed on all white blood cells. The first reported ligand for E-selectin is the sialylated Lewis X (sLe^x). The ligands for L-selectin are carbohydrate moieties (sulfated Le^x) of mucin-like vascular addressins (CD34, GlyCAM-1, MAdCAM-1), which are expressed on the surface of vascular endothelial cells in HEV (Aplin, Howe et al. 1998; Janeway 2005). Selectins take part both in recruiting leukocytes into inflamed sites and in lymphocyte homing to specific tissue. Naive lymphocytes enter lymphoid organs due to interactions between vascular addressins and L-selectins. During the rolling along the blood vessel wall carbohydrate groups presented on proteins located on leukocytes bind to external lectin-like domain of the selectins. This process slows the cell allowing it to leave the blood vessel and enter the site of infection (Kelly, Hwang et al. 2007). The characteristic "rolling" action attributed to leukocytes during the leukocyte adhesion cascade is allowed by the low affinity nature of selectins as well as by the fact that this adhesion receptor is to be shed from the cell surface as soon as the binding occurs (Spertini, Callegari et al. 1994).

To enable the cell to attach firmly and further to cross the barrier of endothelial cells, integrins and molecules of immunoglobulin superfamily are required.

1.6.3.2 Integrins

Integrins are a family of cell-surface glycoproteins that act as receptors for membrane-bound counter-receptors on other cells and on extracellular matrix. They are cell surface receptors which define also cellular shape, mobility, and regulate the cell cycle (Aplin, Howe et al. 1998).

The main two functions of integrins are: attachment of the cell to the ECM and signal transduction from the ECM to the cell. They also play an important role in cell migration (Tarone, Hirsch et al. 2000). During the attachment of the cell the adhesion complexes are formed. These complexes consist of integrins and cytoplasmic proteins which are attached to the actin cytoskeleton. Among the ECM ligands of integrins are fibronectin, vitronectin,

collagen and laminin. Migrating cell creates new attachments to the substrate at its front and at the same time releases those already passed (Lamalice, Le Boeuf et al. 2007). Integrins which are released from the substrate, are taken back into the cell by endocytosis. Next, they can be transported through the cell to its front by the endocytic cycle, where they are added back to the cell surface (Caswell and Norman 2006).

Name	Synonyms	Distribution	Ligands
$\alpha_1\beta_1$	CD49a/CD29	Many	Collagens, laminins.
$\alpha_2\beta_1$	CD49b/CD29	Many	Collagens, laminins
$\alpha_4\beta_1$	VLA-4 (CD49d/CD29)	Hematopoietic cells, leukocytes	Fibronectin, VCAM-1
$\alpha_5\beta_1$	fibronectin receptor (CD49e/CD29)	Widespread	fibronectin and proteinases
$\alpha_6\beta_1$	laminin receptor (CD49f/CD29)	Widespread	matrix macromolecules laminins
$\alpha_L\beta_2$	LFA-1 (CD11a/CD18)	T-lymphocytes	ICAM-1, ICAM-2
$\alpha_M\beta_2$	MAC-1, CR3, CD11b/CD18	Neutrophils and monocytes	Serum proteins, ICAM-1
$\alpha_{IIb}\beta_3$	CD41/CD61	Platelets	fibrinogen, fibronectin
$\alpha_v\beta_3$	vitronectin receptor CD51/CD61	activated endothelial cells, melanoma, glioblastoma	vitronectin, fibronectin, fibrinogen, osteopontin, Cyr61
$\alpha_v\beta_5$	CD51	widespread, esp. fibroblasts, epithelial cells	vitronectin and adenovirus
$\alpha_v\beta_6$	CD51	proliferating epithelia, esp. lung and liver	fibronectin; TGF- β
$\alpha_6\beta_4$	CD49f/CD104	Epithelial cells	Laminin

Table 2. The most important integrins (Smith, Zhu et al. 1995; Aplin, Howe et al. 1998; Janeway 2005)

There are many types of integrins and cells may have multiple patterns on their surface. Each integrin is a heterodimer consisting of two noncovalently-linked subunits, a large α chain that pairs with a smaller β chain (Ruoslahti 1991; Hynes 1992; Rosales, Gresham et al. 1992; Mettouchi and Meneguzzi 2006). The integrin family of vertebrates

includes at least 18 distinct α subunits and 8 or more β subunits which can associate to form more than 24 distinct integrins (Hynes 1992; Rosales and Juliano 1995). The α/β pairing determines the ligand-binding abilities of the integrin (Aplin, Howe et al. 1998). The best known integrins participating in circulating cell – endothelial cell interactions are: VLA 1-6 (*very late antigen*, beta 1 type), MAC-1 (*macrophage-1 antigen*) and LFA-1 (beta 2 type). It is common for cells to make new receptors on their surfaces, or remove them if they need to alter their ability to respond to the environment. This allows cells to make rapid and flexible responses. The signals the cell receives through the integrin could be involved in cell growth, cell division, cell survival, cellular interaction and communication processes.

1.6.3.3 Immunoglobulin-like cell adhesion molecules (Ig CAMs)

Ig CAMs are either homophilic or heterophilic adhesion molecules, which bind integrins or different IgSF CAMs. To this family belong, among others: NCAM (*neural cell adhesion molecule*), ICAM-1 (CD54; *intercellular cell adhesion molecule-1*), VCAM-1 (*vascular cell adhesion molecule-1*), and PECAM-1 (CD31; *platelet-endothelial cell adhesion molecule-1*) (Aplin, Howe et al. 1998).

ICAM-1 (CD54) and **ICAM-2** (CD102) are constitutively expressed and are present in low concentrations on the membranes of leukocytes and vascular endothelial cells. Upon cytokine stimulation, for example by IL-1 (*interleukin-1*) and TNF- α (*tumor necrosis factor-alpha*), ICAM-1 expression increases. This molecule is a ligand for LFA-1, integrin found on leukocytes (Biedermann 2001; Daxecker, Raab et al. 2002).

VCAM-1 (CD106) is a cell surface sialoglycoprotein expressed by endothelial cells only after stimulation by inflammatory cytokines like: TNF- α , IL-1 and/or IL-4. VCAM-1 is present on both large and small vessels. This molecule contains six or seven immunoglobulin domains. Binding to VCAM-1 enables lymphocytes to migrate through vessel walls. VCAM-1 is an endothelial ligand for VLA-4 of the $\beta 1$ subfamily of integrins, and for integrin $\alpha 4\beta 7$ and promotes the adhesion of lymphocytes, monocytes, eosinophils, and basophils (van Buul, Kanter et al. 2007).

PECAM-1 (CD31) is the molecule expressed by endothelial cells, platelets, macrophages and Kupffer cells, granulocytes, NK cells, T lymphocytes, megakaryocytes, osteoclasts, neutrophils. PECAM-1 in macrophages plays a key role in tissue regeneration and

removing aged neutrophils from the body (Aplin, Howe et al. 1998). In adhesion cascade homotypic PECAM/PECAM as well as heterotypic PECAM/ $\alpha_v\beta_3$ interactions are of special importance (Piali, Hammel et al. 1995).

1.6.3.4 Mucin-like molecules

Mucins are a family of high molecular weight, heavily glycosylated proteins produced by many epithelial tissues in vertebrates. They form a negatively charged layer on endothelial surface. They constitute part of adhesion molecules by themselves or by being part of another adhesion molecules, like in MAdCAM-1 (Aplin, Howe et al. 1998).

CD34 is a 110-kDa heavily glycosylated transmembrane protein. It is mainly present on endothelial cells, stem cells, leukemic cells (Pusztaszeri, Seelentag et al. 2006). Its function is still unclear. CD34 present on endothelial cells is one of the major ligands for L-selectin present on lymphocytes and like this, is taking part in adhesion process, especially when decorated with sialylated and/ or sulphated Le^x epitopes (Puri, Finger et al. 1995; Berg, Mullowney et al. 1998). It was also reported to be up-regulated in angiogenesis. According to Ito *et al.* CD34 fulfills significant role in development of blood vessels both in embryos and in adults (Ito, Nomura et al. 1995). The expression of CD34 is increased during embryonic development and later during wound healing (Schlingemann, Rietveld et al. 1990) and also in tumor growth. CD34 molecule is accepted as an endothelial cell marker (Muller, Hermanns et al. 2002). In fact, it is a marker of early endothelial precursor cell (Khan, Solomon et al. 2005).

CD43 (leukosialin) is an adhesion molecule with the NH-terminus heavily glycosylated. The extracellular region of CD43 is called leukosialin and binds SigLec-1 (*sialic acid Ig superfamily lectin molecule-1*) during leukocyte-endothelium interactions (Fukuda and Carlsson 1986; van den Berg, Nath et al. 2001). It is present on tissue macrophages, dendritic cells, smooth muscle cells, epithelium and endothelium (McEvoy, Jutila et al. 1997; Rupniewska, Rolinski et al. 2000). CD43 is postulated to be responsible for regulating first contacts between these cells (Ostberg, Barth et al. 1998).

1.6.3.5 Chemokines

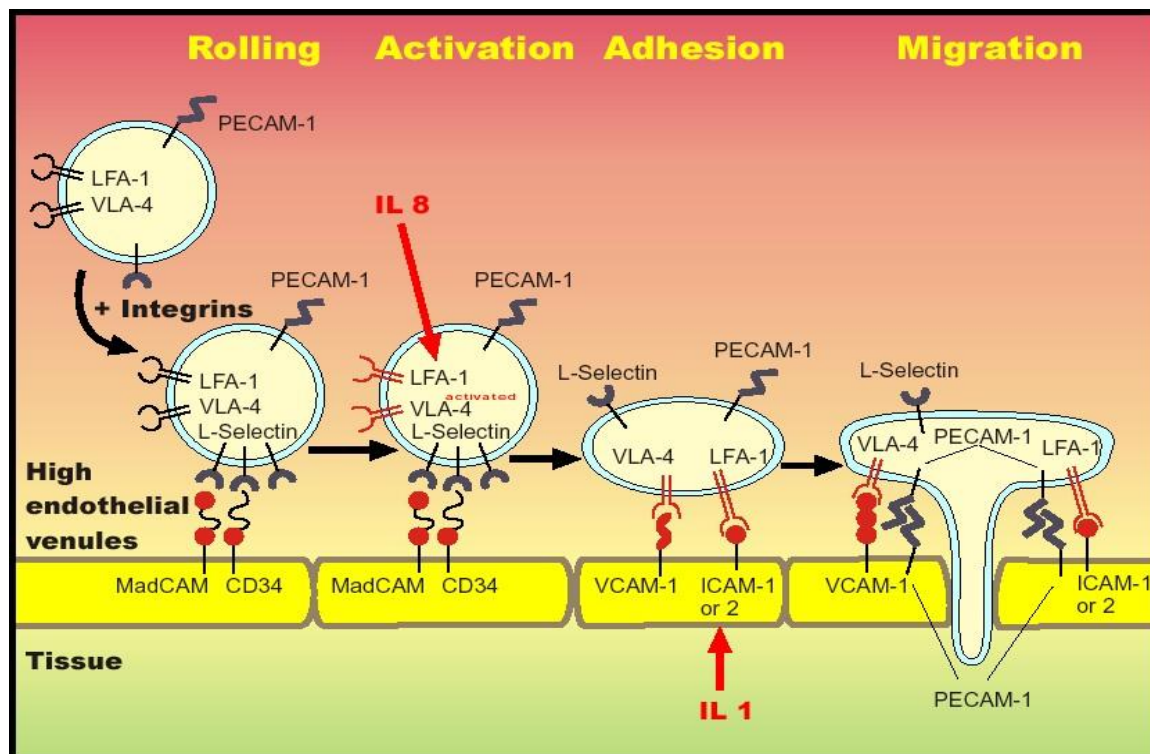
Chemokines are the molecules that guide circulating cells to the adhesion place. The migrating cells follow the signal of increasing chemokine concentration (Laing and Secombes 2004). As chemotactic cytokines, chemokines “drive” the migration and adhesion of leukocytes, monocytes, neutrophils and other circulating cells. In humans, some chemokines, eg. SDF-1 alpha or MIP-3alpha, trigger the adhesion of peripheral lymphocytes, but others, like TECK or CTACK mediate memory lymphocytes trafficking to small intestine and skin, respectively (Duś, Paprocka, Kieda, 2003).

Chemokine	Receptor	Cells attracted	Major effect
Cxcl1 (Gro1, Mgsa, Growth-regulated alpha protein precursor)	Cxcr2	Neutrophils, naive T cells, fibroblasts	Activation of neutrophils, angiogenesis, inflammation, wound healing
Cxcl2 (Gro 2, Mip 2, Macrophage inflammatory protein 2 precursor,	Cxcr2	Neutrophils, naive T cells, fibroblasts	Activation of neutrophils, fibroplasia, angiogenesis
Cxcl9, (MIG)	Cxcr3	Lymphocytes, NK cells	T cell trafficking
Cxcl10, (IP10, Crg-2)	Cxcr3	Resting T cells, NK cells, Monocytes	Immunostimulation, Antiangiogenesis Promotion of T _H 1 immunity
Cxcl12 (SDF-1)	Cxcr4	Naive T cells, Progenitor (CD34 ⁺) B cells,	B-cell development, Lymphocyte homing, Competition with HIV-1
Ccl2 (MCP-1, JE)	Ccr2B	Monocytes NK and T cells	Activation of macrophages, Basophils histamine release, Promotion of T _H 2 immunity
Ccl3 (MIP-1 α)	Ccr1, Ccr3 Ccr5	Monocytes NK and T cells Basophils Dendritic cells	Antiviral defence, Promotion of T _H 1 immunity, Competition with HIV-1
Ccl4 (MIP-1 β)	Ccr1, Ccr3 Ccr5	Monocytes, NK and T cells Dendritic cells	Competition with HIV-1
Ccl5 (RANTES)	Ccr1, Ccr3 Ccr5	Monocytes, NK and T cells, Basophils, Eosinophils, Dendritic cells	Degranulation of basophils, Activation of T cells, Chronic inflammation

Table 3. Chemokines and their receptors expressed by endothelial cells

Additionally, chemokines play a crucial role in integrin activity, changing their avidity and clustering. Chemokines are produced as pro-peptides and are cleaved during secretion to produce an active mature protein (Laing and Secombes 2004). Depending on the arrangement of the two first cysteins, chemokines are categorized into four subfamilies: CXC (α), CC (β), C (γ) and CX₃C (δ). Similarly to GIRK2 protein, chemokines function by activating G protein coupled receptors. It was also found that they are involved in angiogenesis regulation (Laing and Secombes 2004).

Cell adhesion molecules presented above participate in leukocyte extravasation - both naïve and memory lymphocytes undergoing homing and recirculation as well as leukocytes movement out of the circulatory system, towards the site of tissue damage or infection. Leukocyte extravasation occurs mainly in post-capillary venules in secondary lymphoid tissues, where haemodynamic shear forces are reduced and where the adhesion molecules of HEV are stopping specifically the proper lymphocytes to allow their extravasation (Ley, Laudanna et al. 2007).



http://www.med.uni-magdeburg.de/fme/institute/iim/engl/kliche_ef.html

Figure 1. 6. Leukocytes adhesion and extravasation

Leukocyte extravasation is often presented as an adhesion cascade - sequence of adhesion and activation events, which ends with leukocyte extravasation (Fig. 1.6). There are at least four steps of the adhesion cascade: weak adhesion-rolling, activation, strong adhesion, and final migration/transmigration. At any given moment rolling, activation, strong adhesion, and migration/transmigration happen in parallel, involving different leukocytes in the same microvessel. Often, especially in the case of leukocyte adhesion, initial chemoattraction step is necessary (Janeway 2005).

1.6.3.6 Chemoattraction

Upon recognition of and activation by pathogens, resident macrophages in the affected tissue release cytokines such as IL-1, TNF- α and chemokines. IL-1 and TNF- α induce the endothelial cells of blood vessels near the site of infection to express cellular adhesion molecules, mainly selectins. Circulating leukocytes migrate toward the site of injury or infection due to the presence of chemokines (Johnson-Leger and Imhof 2003).

1.6.3.7 Rolling

Carbohydrate ligands on the circulating leukocytes bind to selectin molecules on the inner wall of the vessel, with marginal affinity, in a reversible way. The initial adhesion of circulating lymphocytes to vascular endothelium is induced by mutual interactions of lymphocyte homing receptors with an array of endothelial cell ligands or counter-receptors (GlyCAM-1 or MAdCAM-1). This causes the leukocytes to slow down and to begin rolling along the inner surface of the vessel wall. During this rolling motion, transitory bonds are formed and broken between selectins and their ligands (Kubes 2002; Johnson-Leger and Imhof 2003; Ley, Laudanna et al. 2007).

1.6.3.8 Activation

During this stage, chemokines released by macrophages activate the rolling leukocytes and cause surface integrin molecules to switch from the default low-affinity state to a high-affinity state. This is assisted through juxtacrine activation of integrins by chemokines

and soluble factors released by endothelial cells. Chemokines are recognized by G protein-coupled receptors on leukocytes. This results in the activation of integrin molecules. In consequence, the leukocyte attaches firmly to the endothelium and the rolling is stopped (Kubes 2002; Johnson-Leger and Imhof 2003; Ley, Laudanna et al. 2007).

1.6.3.9 Tight adhesion

In the activated state, integrins bind tightly to complementary receptors expressed on endothelial cells, with high affinity. This causes the immobilization of the leukocytes, despite the shear forces of the ongoing blood flow. The firm adhesion step requires interactions between the leukocyte integrins (LFA-1, MAC-1) and ICAMs molecules presented on the endothelium (Kubes 2002; Johnson-Leger and Imhof 2003; Ley, Laudanna et al. 2007).

1.6.3.10 Transmigration

The cytoskeleton of the leukocyte is reorganized in such a way that the leukocytes are spread out over the endothelial cells. In this form, leukocytes extend pseudopodia and pass through gaps between endothelial cells. Transmigration of the leukocyte occurs when PECAM-1 proteins, found on the leukocyte and endothelial cell surfaces, interact and effectively pull the cell through the endothelium. The leukocytes secrete also proteases that degrade the basement membrane, facilitating them to escape the blood vessel – a process known as diapedesis. Once in the interstitial fluid, leukocytes migrate along a chemotactic gradient toward the site of injury or infection (Kubes 2002; Johnson-Leger and Imhof 2003; Hordijk 2006; Ley, Laudanna et al. 2007).

Following firm adhesion the cells begin to extravasate (in a process called diapedesis) through the endothelium between adjacent endothelial cells. This step also involves the leukocyte integrins (LFA-1 and MAC-1) as well as PECAM-1 which is expressed at the intercellular adhesive junctions of endothelial cells. These interactions enable the leukocytes to squeeze between the endothelial cells and penetrate the surrounding target tissue, inflamed tissue or lymph node (Gołab 2004; Janeway 2005).

AIM OF STUDY

One of the main disturbances in Down syndrome concerns the biology and function of the endothelium. Endothelial cells are proven to significantly participate in circulating cells homing through the panel of adhesion molecules and secreted cytokines. Although *GIRK2* (G-protein inwardly rectifying potassium channel) is one of many genes involved in DS pathology, it is hypothesized that additional *GIRK2* copies could affect numerous aspects of endothelial cells biology. Our hypothesis states that the altered endothelial cells may be the reason of several immunological disturbances, such as disturbed proportions of T lymphocytes population in transgenic YAC67 and YAC84 mice.

The aim of this study was to investigate the biology and function of endothelial cells isolated from transgenic mice YAC67 and YAC84 possessing additional copies of *GIRK2* gene in comparison to the endothelial cells isolated from background FVB mice. Special attention has been focused on the adhesive interactions as well as the angiogenic abilities of transgenic endothelial cells.

Detailed knowledge concerning the changes in endothelial cells biology in mice YAC67 and YAC84 would give additional information of the general mechanism of Down syndrome pathology and may help in searching pharmacological solution eliminating at least some of Down syndrome effects.

2. MATERIALS AND METHODS

2.1 ANIMALS

Mice were obtained from CDTA CNRS (Centre de Développement des Techniques Avancées-Centre National de la Recherche Scientifique) – (Orleans, France). The animals were bred and kept in routine SPF conditions.

Mice:

FVB – an inbred nontransgenic mouse strain, preferable for transgenic analyses;

YAC67 and YAC84 - transgenic FVB mice, with incorporated YAC 285E6 (yeast artificial chromosome 285E6) were created by insertion of fragment of human chromosome 21 into the YAC. Inserted fragment contain only one whole gene – *GIRK2* . Heterozygous lines (YAC67 and YAC84) were created by Smith *et al.* by microinjection of YAC 285E6 (Smith, Zhu et al. 1995). Homozygous animals were selected by Lignon *et al.* (Lignon, Bichler et al. 2008). Line YAC67 contains 2 copies of YAC 285E6, it is assumed that mice from line YAC84 have 4 copies of YAC 285E6 (Smith, Zhu et al. 1995; Smith and Rubin 1997), (Lignon personal communication).

2.2 CELL LINES

T- and B-cell lymphomas used for adhesion tests:

EL4 – C57BL/6N mouse T-cell lymphoma (ATCC TIB-39)

EL4.IL-2 – EL4 subline with constitutive expression of IL2 and IL2R (ATCC TIB-181)

RAW 8.1 – BALB/c mouse B-cell lymphoma (ATCC TIB-50)

2.3 ISOLATION AND CULTURE OF MICROVASCULAR ENDOTHELIAL CELLS

Isolation and culture of mouse ECs were performed according to the previously established technique (Bizouarne, Denis et al. 1993).

The bone marrow, thymus, peripheral lymph nodes, Peyer's patches and brain were collected and treated with betadine (ASTA Medica, Merignac, France) diluted 1:10 (v/v) in complete phosphate buffered saline (c-PBS, PBS supplemented with 1mM CaCl₂ and 0.5 mM MgCl₂) for 10 min., at room temperature (RT) and washed after with c-PBS. The tissue samples were cut into small pieces with surgical blades, washed with medium and

digested for 30 min., at 37°C, with a type 1 collagenase solution (5 mg/mL; Gibco BRL, Cergy Pontoise, France). Then cells were washed three times with c-PBS and cell cultures were started in Primaria® dishes (Falcon, Becton Dickinson, Grenoble, France). Primary cultures were grown in RPMI 1640 (Gibco BRL, Cergy Pontoise, France) supplemented with 10% of FBS (fetal bovine serum) and antibiotics: 40 µg/ml gentamicin (Gibco/Fisher) and 0.5 µg/ml Fungizone (Gibco/Fisher) (complete growth medium). After 1 to 2 hr, non adherent cells were removed and submitted to a second adhesion step. Subconfluent primary cultures were resuspended in growth medium and plated into 25 cm² flasks.

2.4 IMMORTALIZATION AND SELECTION OF ENDOTHELIAL CELL LINES

Immortalization and selection of mouse endothelial cell lines was performed according to the technique established previously by Kieda and co-workers (CNRS patent No. 99-16169); (Kieda, Paprocka et al. 2002).

Exponentially growing endothelial cells (ECs) cultures were transfected with the pSV3-neo plasmid (ATCC; American Type Culture Collection, Rockville, MD), with the complete SV40 early region of the large T-antigen gene and the neo^R gene (Cavender, Conn et al. 1995; Reddel, De Silva et al. 1995). Transfection was performed as previously described (Bizouarne, Denis et al. 1993; Bizouarne, Mitterrand et al. 1993). Lipofectin (1-10 µg/mL) and plasmid (1-6 µg/mL) were mixed together in serum- and antibiotic-free OptiMEM. ECs cultures were incubated with the complex-containing solution for 24 hr. The cell cultures were further allowed to grow for 7 to 14 days before starting geneticin selection (G418, 10 - 200 µg/mL, Gibco BRL, Cergy Pontoise, France). G418 resistant cells were frozen and stored in liquid nitrogen.

After defreezing the cells were washed by centrifugation and grown routinely in OptiMEM (Gibco BRL, Cergy Pontoise, France) supplemented with FBS (2%, v/v; Dutcher, Brumath, France) and antibiotics (complete growth medium). Adherent cells were passaged using of 0.25% trypsin (Gibco, BRL, Cergy Pontoise, France), 0.05% EDTA (Ethylene-diamine-tetra-acetic acid) (w/vol) solution in phosphate buffered saline (PBS). For immunocytochemistry studies, cells were detached from culture vessel with type 1 collagenase (Gibco, BRL, Cergy Pontoise, France) (0.5 mg/mL)/BSA (bovine serum albumin) (50 mg/mL) solution in c-PBS, for 5 min. in 37°C. Collagenase was further blocked using 0.5% BSA/c-PBS solution, cells were washed and resuspended in c-PBS.

2.5 MURINE LYMPHOCYTES ISOLATION

From mice FVB thymuses or spleens were collected. The organs were minced with scissors and gently pressed through a mesh screen to obtain a cell suspension in RPMI 1640 medium. Cells were washed and frozen in RPMI 1640 plus 40% fetal bovine serum and 10% DMSO (dimethyl sulfoxide). After defreezing process the cells were washed with c-PBS and resuspended in RPMI 1640 plus 2% fetal bovine serum.

2.6 ANTIBODIES

Anti-mouse monoclonal antibodies: rat CD29, rat CD34 and rat CD43 (BD Pharmingen, Le Pont de Claix, France), rat CD133 (eBioscience Montrouge, France), polyclonal antibodies rabbit anti-mouse: ACE and vWf (Santa Cruz Biotechnology Inc, Bergheimer, Heidelberg, Germany). FTC (fluorescein thiocarbamyl)-conjugated rat anti-mouse CD3 and PE (Phycoerythrin)-conjugated rat anti-mouse CD19 antibodies were purchased from BD Pharmingen.

As isotypic controls rat immunoglobulins (Sigma ImmunoChemicals, France) and rabbit immunoglobulins (Sigma Aldrich, France) were used.

As secondary antibodies, FTC-conjugated anti-rat immunoglobulins (ZYMED Laboratories INC, USA) and FTC-conjugated goat anti-rabbit immunoglobulins (Sigma, France) were used.

2.7 FLOW CYTOMETRY ANALYSIS

Endothelial cells were washed with c-PBS/0,5%BSA/0,1% NaN₃ solution. Then the cells were detached from the plate by type 1 collagenase solution. Half of the cells were permeabilised with 0.2% saponin (Sigma, l'Isle d'Abeau Chesnes, Saint Quentin Fallavier, France) / 2% paraformaldehyde (Merck-Schuchardt, Germany) (w/vol) solution in c-PBS for 10 min at 37°C and washed by c-PBS containing saponin (0.2%, w/vol).

Fresh and permeabilised cells were incubated with anti-mouse CD29, CD34, CD43 and CD133 rat monoclonal antibodies for 30 min. at 37°C (0.3µg/3x10⁵ cells, 5µg/mL). As an isotypic control rat IgG was used. Labeled cells were fixed with 2% paraformaldehyde

(Merck-Schuchardt, Germany) (w/vol) for 10 min. at 37°C. As a secondary antibody the goat FTC-anti-rat immunoglobulin F(ab')₂ (10 µg/mL), was used for 30 min. at 4°C.

Fluorochrome labeled cells were analyzed by flow cytometry using FACS LSR (Becton Dickinson, Sunnyvale Ca, USA). Five thousand events, gated by forward and side scatters, were acquired and analyzed using the CellQuest software (Becton Dickinson). Mean fluorescence intensity of the appropriate control cells was set below 10 and this value was used as a threshold for positive cells.

2.8 INTRACELLULAR VON WILLEBRAND FACTOR (VWF) AND ANGIOTENSIN-CONVERTING ENZYME (ACE) DETECTION

Detection of vWf and ACE on mouse endothelial cell lines was performed according to the technique established previously by Kieda and co-workers (Kieda, Paprocka et al. 2002).

Cells were grown on eight-well gelatin-coated microscope slides (ICN Biomedicals, Aurora, OH, USA) for 48 hr. Cells were permeabilised for 10 min. with 0.2% (w/vol) saponin Sigma (l'Isle d'Abeau Chesnes, Saint Quentin Fallavier, France) and 2% (w/vol) paraformaldehyde in c-PBS solution. Then rabbit anti-mouse vWf or ACE polyclonal antibody (50 µg/mL) in c-PBS with 0.5% BSA was applied to the cells for 1 hr at 37°C. Control cells were treated with rabbit normal serum (50 µg/mL) in c-PBS with 0.5% BSA. The secondary antibody was goat (FTC)-anti-rabbit immunoglobulin (12.5 µg/mL), and cells were incubated with this reagent for 1 hr at 37°C, and further examined by fluorescence microscopy and phase contrast inverted microscope DIC equipped (Zeiss, Axiovert 200).

2.9 ADHESION ASSAY

Endothelial cells were seeded on 24 well tissue culture plates (Falcon, Dutscher, France) and adhesion test was performed when cells reached subconfluency, usually after 48 hr of culture. The EL4, EL4.IL-2 and RAW8.1 lymphocytes ($\sim 2 \times 10^7$) were labeled with 7 µL of PKH26-GL red dye diluted in 1 mL of diluent C (Sigma, l'Isle d'Abeau Chesnes, France) according to the manufacturer's instructions. Labeling was stopped by washing once with c-PBS/0,5% BSA and then three times with c-PBS/0.1% BSA. The quantity of endothelial cells per well was counted and the lymphocytes were overlaid onto

EC monolayers in a ratio of 5 lymphocytes per 1 endothelial cell. After 20 min. of incubation at 37°C, non-adherent lymphocytes were removed by washing with c-PBS. The adherent lymphocytes and ECs were collected from the plates by EDTA (0,5mM) treatment. The number and type of adhered lymphocytes and ECs were assessed by flow cytometry to quantify a ratio expressing the number of lymphocytes adhering per EC.

In the case of lymphocytes isolated from mice FVB, after cells detachment cells were labeled with anti-CD3-FTC (T cell specific) and anti-CD19-PE (B cell specific) antibodies to identify the lymphocyte population. Then cells were assessed by flow cytometry as described above (Paprocka, Dus et al. 2008).

2.10 PSEUDOVESSELS FORMATION ASSAY

Endothelial cells in 400 µl of OptiMEM supplemented with 2% FBS were seeded on 24 well plates on 70 µl of previously polymerized Matrigel™ BD Pharmingen (Le Pont de Claix, France) diluted (2:3) in cold OptiMEM and the tube formation assay was performed for 24 hr at 37°C. The culture morphology and pseudovessels formation were captured with a phase-contrast microscope and photographed after 5, 12 and 24 hr of culture. The control cells were seeded, at the same concentrations, on plastic 24 well tissue culture plates without Matrigel™.

Matrigel™ is an extract from EHS (Engelbreth-Holm-Swarm) mouse sarcoma, which contain the components present in the basement membrane and which is biologically active. Matrigel™ promotes cell differentiation and can be used to study the invasiveness of the tumor cells. It is able to form 3D gel at 37°C (Kleinman and Martin 2005).

The main components of Matrigel™ are proteins like laminin, collagen IV and entactin. Apart from the proteins Matrigel™ contains also various growth factors such as: TGF-β (*transforming growth factor β*), EGF (*epidermal growth factor*), IGF-1 (*insulin-like growth factor-1*), bFGF, and PDGF (*platelet-derived growth factor*) which promote differentiation and proliferation of many cell types. Matrigel™ contains also many other proteins in small amounts and its exact composition is not known (Vukicevic, Kleinman et al. 1992; Kleinman and Martin 2005).

2.11 cDNA MICROARRAY ANALYSIS OF GENE EXPRESSION PROFILE

DNA chips hybridization was performed on DNA chips manufactured in Genopole® CEA microarray platform (Evry, France).

RNA was isolated from subconfluent EC culture having grown for 48 hr, using RNeasy mini kit (Qiagen, Courtaboeuf, France). The RNA concentration was measured by NanoDrop ND-1000 (Labtech, Palaiseau, France). The integrity of isolated RNA was verified using RNA Nano Chips and Agilent 2100 bioanalyser (Agilent Technologies, Waldbronn, Germany). All procedures were performed according to the manufacturer's instructions.

In order to obtain a sufficient amount of material, 500 ng of each RNA sample were amplified by using the Amino Allyl MessageAmp™ kit (Ambion, Courtaboeuf, France).

Amplification was performed according to the manufacturer instructions and it comprises:

- synthesis of first strand cDNA,
- synthesis of second strand cDNA,
- cDNA purification,
- in vitro transcription in order to obtain aRNA (amplified RNA)
- aRNA purification (Fig. 2.1).

The concentration of amplified RNA was assessed by NanoDrop ND-1000 measurement.

In order to compare the gene expression of examined samples with references, RNA labeling was performed. As the references the mix of RNA isolated from each cell culture was prepared (12 µg of RNA taken from each sample and mixed). 15 µg of each sample were labeled with Cy3 Post-Labeling Reactive Dye Pack (GE Healthcare Life Sciences, Buckinghamshire, England). The mixed references solution was dispatched to the separate tubes in order to obtain 15 µg of RNA/tube and labeled with Cy5 Post-Labeling Reactive Dye Pack (GE Healthcare Life Sciences, Buckinghamshire, England). To prevent the fluorescent signal decrease, all the incubations were performed in the dark.

The concentration of labeled RNA and the quality of labeling were verified using NanoDrop ND-1000.

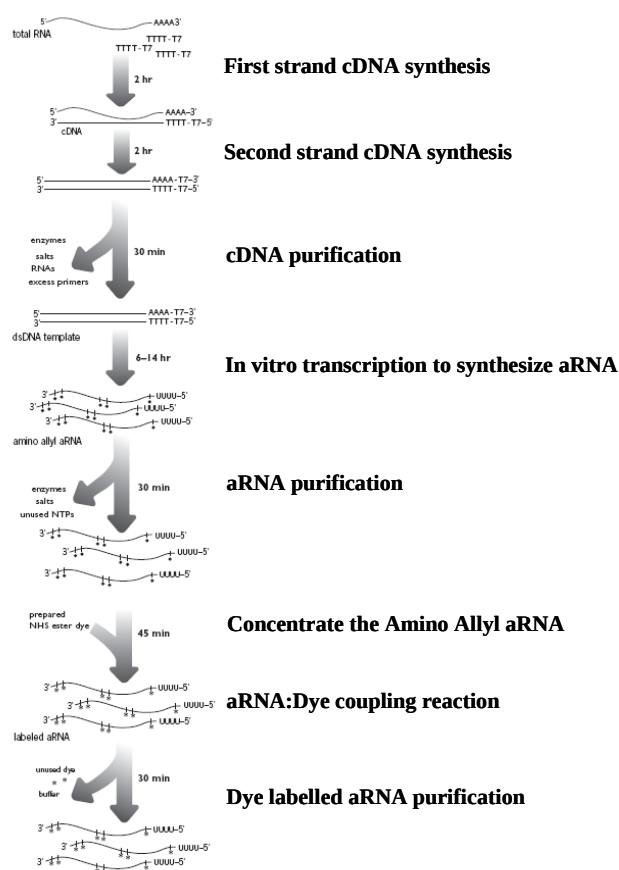


Figure 2.1. Amino Allyl MessageAmp™ Procedure (from Instruction manual, Amino Allyl MessageAmp™)

After labeling, 1 µg of each sample was mixed with 1 µg of the reference. The prepared RNA mixture was brought up to 9 µL with nuclease-free water. 1 µL of 10x Fragmentation Buffer (Ambion, Courtaboeuf, France) was added to each sample. The RNA fragmentation was performed at 70°C for 15 min. in a heating block. The reaction was stopped by adding 1 µL of the Stop Solution (Ambion, Courtaboeuf, France).

After fragmentation, RNA was dried under vacuum and dissolved in 42 µL of hybridization solution

	<u>final concentration</u>
Denhardt 50x	5x
SSC 20x (sodium citrate, Eurobio, Les Ulis, France)	3.5x
SDS (Sodium dodecyl sulfate) 10%	0.3%
Yeast RNA 10 µg/µL	0.5 µg/µL
PolyA 10 µg/µL	0.5 µg/µL
H ₂ O	50%

Then, denaturation step was performed by heating samples at 98°C for 2 min., and finally, a preannealing step occurred at 37°C for 30 min.

Chips preparation before hybridization:

The aim of this step is to obtain the best fixation of oligonucleotides to the chips. To rehydrate the chips, they were placed in highly humid atmosphere for 2 hr (incubator 42°C, 80% of humidity), next were left for 2 hr at room temperature to dry. Then, the chips were placed in a blocking solution (150 μ M Ethanolamine (Sigma) in 50 mM NaBorate, pH 9 (Sigma)) for 1 hr at room temperature. Finally, the chips were washed during 1 min. in water and dried by centrifugation (3min., 1200 rpm).

After the preannealing step, 40 μ L of each sample were pipetted onto the chips. Hybridization was performed at 42°C for 17 hr using a Glass Array Hybridization Cassette, (Ambion, Courtaboeuf, France). Chips were washed in the following washing solutions: 2x SSC, 0,1% SDS, 1x SSC 0,2x SSC, 0,05x SSC and dried by centrifugation (3min., 1200 rpm).

Image acquisition and results analysis

Hybridized arrays were scanned at 10 μ m/pixel resolution by fluorescence confocal microscopy (ScanArray Express; GSI-Lumonics, Billerica, MA). Signal intensities were extracted with Genepix Pro 5.0 image analysis software (Axon Instruments, Sunnyvale, CA). The average of the resulting total Cy3 and Cy5 signal gives a ratio that is used to balance and normalize the signals. The signals to calculate Cy3: Cy5 ratios were calculated by subtraction of background from the elementary total signal.

Data normalization and analysis were performed using GeneSpring version 6.0 (Silicon Genetics, Redwood City, CA).

Normalization procedure:

A Lowess curve was fit to the log-intensity versus log-ratio plot. 20% of the data was used to calculate the Lowess fit at each point. This curve was used to adjust the control value for each measurement. If the control channel was lower than 10 then 10 was used instead. Each gene was divided by the median of its measurements in all samples. If the median of the raw values was below 10 then each measurement for this gene was divided by 10 if the numerator was above 10, otherwise the measurement was not taken into account.

3. RESULTS

3.1 ESTABLISHMENT OF MOUSE ENDOTHELIAL CELL LINES

Heterozygous transgenic mice were established by microinjection of the YAC 285E6 into FVB zygotes (Smith, Zhu et al. 1995; Smith, Stevens et al. 1997). Homozygous animals were selected with a conventional test cross based on three generations (Lignon, Bichler et al. 2008).

Endothelial cells were isolated from homozygous transgenic mice: YAC67, YAC84 with incorporated human *GIRK2* gene inserted in yeast artificial chromosome 285E6 (YAC 285E6). Homozygous mice YAC67 contain 2 copies of YAC 285E6; mice YAC84 are expected to contain 4 copies (Lignon personal communication).

Endothelial cells were isolated from the primary lymphoid organs (bone marrow, thymus) responsible for lymphocyte maturation, from secondary lymphoid organs (peripheral lymph nodes, Peyer's patches) taking part in lymphocyte education and from the brain (Table 4).

To obtain the control, endothelial cells were also isolated from the background FVB mice from all listed organs.

	FVB	YAC67	YAC84
Bone marrow	mBMMEC FVB	mBMMEC YAC67	mBMMEC YAC84
Thymus	mThMEC FVB	mThMEC YAC67	mThMEC YAC84
Peripheral lymph nodes	mPLNMEC FVB	mPLNMEC YAC67	mPLNMEC YAC84
Peyer's patches	mPPMEC FVB	mPPMEC YAC67	mPPMEC YAC84
Brain	mBrMEC FVB	mBrMEC YAC67	mBrMEC YAC84

Table 4. Mouse endothelial cells isolated from control (FVB) and transgenic (YAC67, YAC84) mice.

mBMMEC – mouse bone marrow microvascular endothelial cells; mThMEC – mouse thymus marrow microvascular endothelial cells; mPLNMEC – mouse peripheral lymph nodes microvascular endothelial cells; mPPMEC – mouse Peyer's patches microvascular endothelial cells; mBrMEC – mouse brain microvascular endothelial cells.

All isolated organs were treated as described in materials and methods section and cultures were started from exponentially growing primary endothelial cells (ECs) that were further transfected with the pSV3-neo plasmid. The cell cultures were allowed to grow for 7 to 14 days, before selection by geneticin.

In order to verify if the isolated cells were indeed from endothelial origin it was necessary to characterize the obtained lines by the general markers that an endothelial cell possess. In the further work the estimation and distribution of the specific cells markers that might identify the organospecificity and particularity of the transgenic cells were compared to the controls.

3.2 THE PRESENCE OF GENERAL MARKERS OF ENDOTHELIAL CELL LINES: VWF AND ACE

Von Willebrand factor (vWf) and angiotensin converting enzyme (ACE) are the main markers of endothelial cells. vWf is stored in Weibel-Palade bodies located in the cytoplasm of endothelial cells (Vischer and de Moerloose 1999). ACE is a large glycoprotein spanning the cell membrane (Dzau, Bernstein et al. 2002).

To confirm that the phenotype of isolated cells is an endothelial type, the presence of vWf (Fig. 3.1 A) and ACE (Fig. 3.1 B) was evaluated by fluorescence microscopy. Permeabilized cells were labeled with anti-vWf and anti-ACE antibodies. All cell lines were vWf- and ACE- positive. Figure 3.1 A presents labeling with anti-vWf antibody of mThMEC YAC84, which was representative for all endothelial cell lines. The labeling appears distributed in a typical manner, detecting the Weibel Palade bodies in all cell lines.

The figure 3.1 B represents the ACE detection and distribution in representative thymus derived EC lines (mThMEC YAC84). ACE is detected in the whole cell population, what confirms the origin of the cells as endothelial.

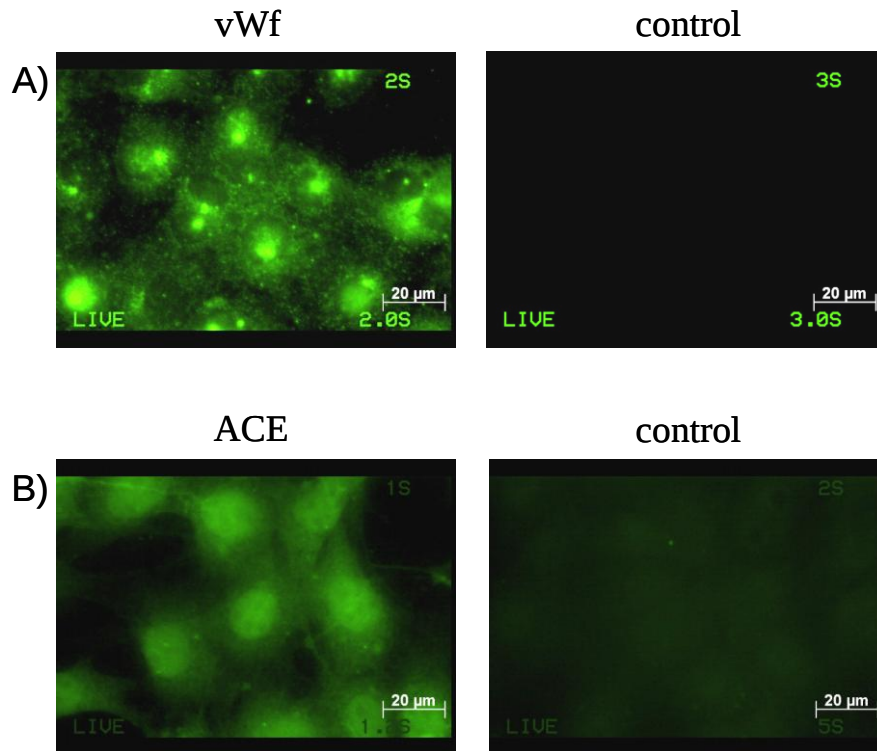


Figure 3.1. Intracellular localization of von Willebrand factor (vWF) and angiotensin converting enzyme (ACE)

After permeabilization (see Materials and Methods) cells were incubated for 1 hr at 37°C with rabbit anti-mouse vWf – von Willebrand factor polyclonal antibody (A) or ACE – angiotensin converting enzyme polyclonal antibody (B), then treated with goat FTC-anti-rabbit immunoglobulin antibody. Control was done in the presence of rabbit normal serum. Fluorescence was evaluated by microscopy (Zeiss, Axiovert 200M). Picture is the representative example (mThMEC YAC84) of the labeling obtained with all endothelial cell lines used in the study.

3.3 CHARACTERIZATION OF THE PHENOTYPE OF TRANSGENIC MOUSE ECS LINES FROM YAC67 AND YAC84 MICE VS NON-TRANSGENIC FVB CELLS

To establish the differences between the phenotype of ECs isolated from transgenic mice (containing additional copies of *GIRK2* gene) versus control ECs phenotype, the expression of cell surface adhesion molecules: CD29 ($\beta 1$ integrin), CD34, CD43 as well as the precursor cell marker CD133 were analyzed by flow cytometry. These markers were chosen because:

- CD29 has been proposed as maintaining the integrity of mature central nervous system vasculature. During maturation, CD29 in the central nervous system vasculature is highly expressed (Stephens, Sutherland et al. 1995);

- CD34 adhesion molecule is one of the major ligands for the L-selectin present on lymphocytes. As such, CD34 takes part in the adhesion process (Puri, Finger et al. 1995);
- CD43 (leukosialin) is an adhesion molecule which takes part in leukocyte-endothelium interactions. CD43 is responsible for regulating the early contacts between cells in the adhesion process (Ostberg, Barth et al. 1998);
- CD133 antigen is an early hematopoietic and endothelial stem cell marker. Therefore, it was utilized to study if transgenic ECs exhibit the phenotype of completely differentiated cells. It was an observation that early endothelial progenitor cells (EPCs) in the bone marrow, or immediately after their migration into the systemic circulation, are positive for CD133 (Hristov, Erl et al. 2003).

The above described established endothelial cell lines were studied.

Figure 3.2 demonstrates CD34 expression that displayed significant differences among the bone marrow derived ECs (Fig. 3.2 A, C) as well as among the thymus-derived ones (Fig. 3.2 B, D). This is visible mainly on the intact cells (Fig. 3.2 A, B) as compared to permeabilized (Fig. 3.2 C, D) suggesting a role in the adhesion/recognition/selection process as our hypothesis was based on.

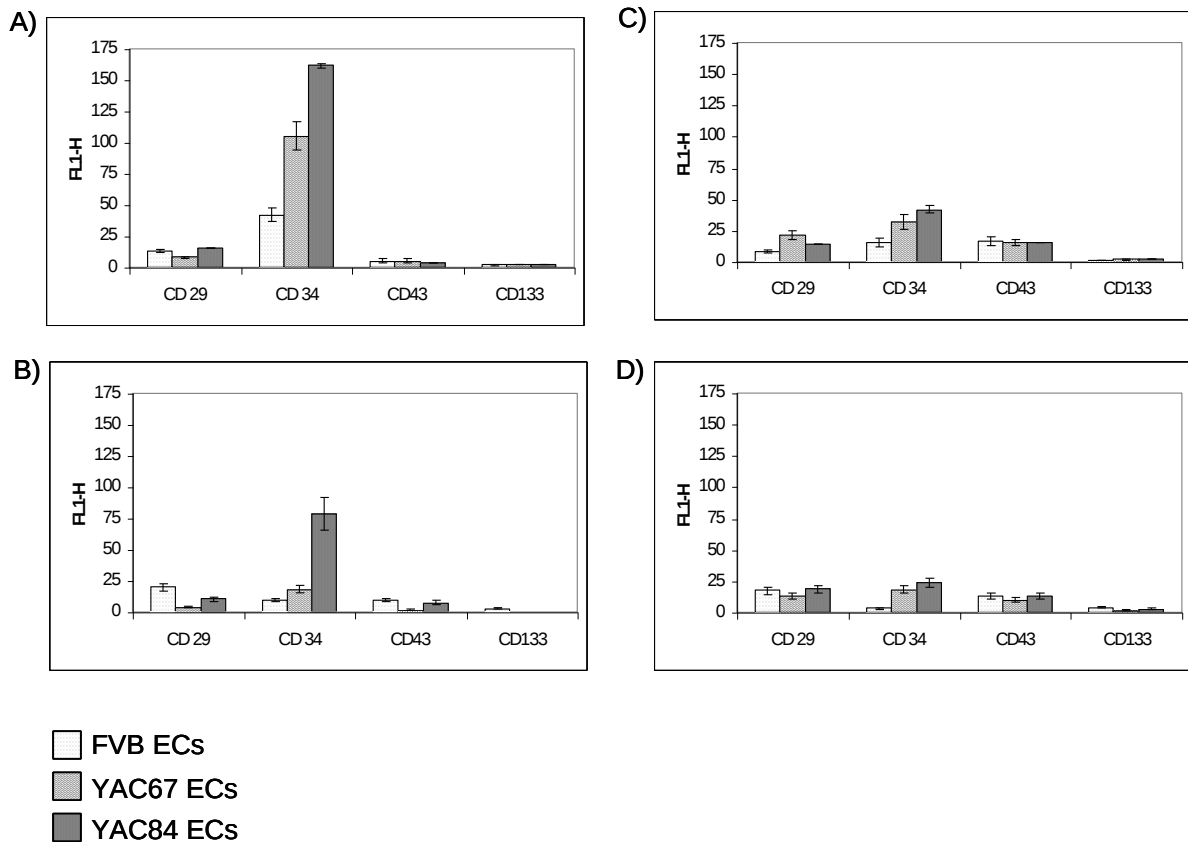


Figure 3.2. Expression of CD29, CD34, CD43 and CD133 molecules on ECs from transgenic and non-transgenic mice

Collagenase-collected, non-permeabilised cells from bone marrow (A) and thymus (B) as well as cells permeabilised from bone marrow (C) and thymus (D) were labeled with rat IgG2a anti-mouse CD29, rat IgG2a anti-mouse CD34 (RAM 34), rat IgG2a anti-mouse CD43, rat IgG1 anti-mouse CD133 and isotypic control further revealed by goat FTC-anti-rat immunoglobulin F(ab')₂. Green fluorescence intensity FL1-H (horizontal axis) represents delta value of the reactivity to the mAb and isotypic control. Graphs represent the mean of 4 experiments analysed by flow cytometry.

Figure 3.3 confirms indeed an organospecificity of the CD34 expression. Although it is less enhanced on the surface of ECs established from secondary lymphoid organs (peripheral lymph nodes (Fig. 3.3 A) and Peyer's patches (Fig. 3.3 B)) from transgenic animals as compared to normal FVB mice than in the case of primary lymphoid organs, it still remains higher.

Noticeably, a very high difference is observed among the CD34 levels of expression on cells derived from brain ECs (Fig. 3.3 C, F). This might be very significant in terms of the blood brain barrier activity.

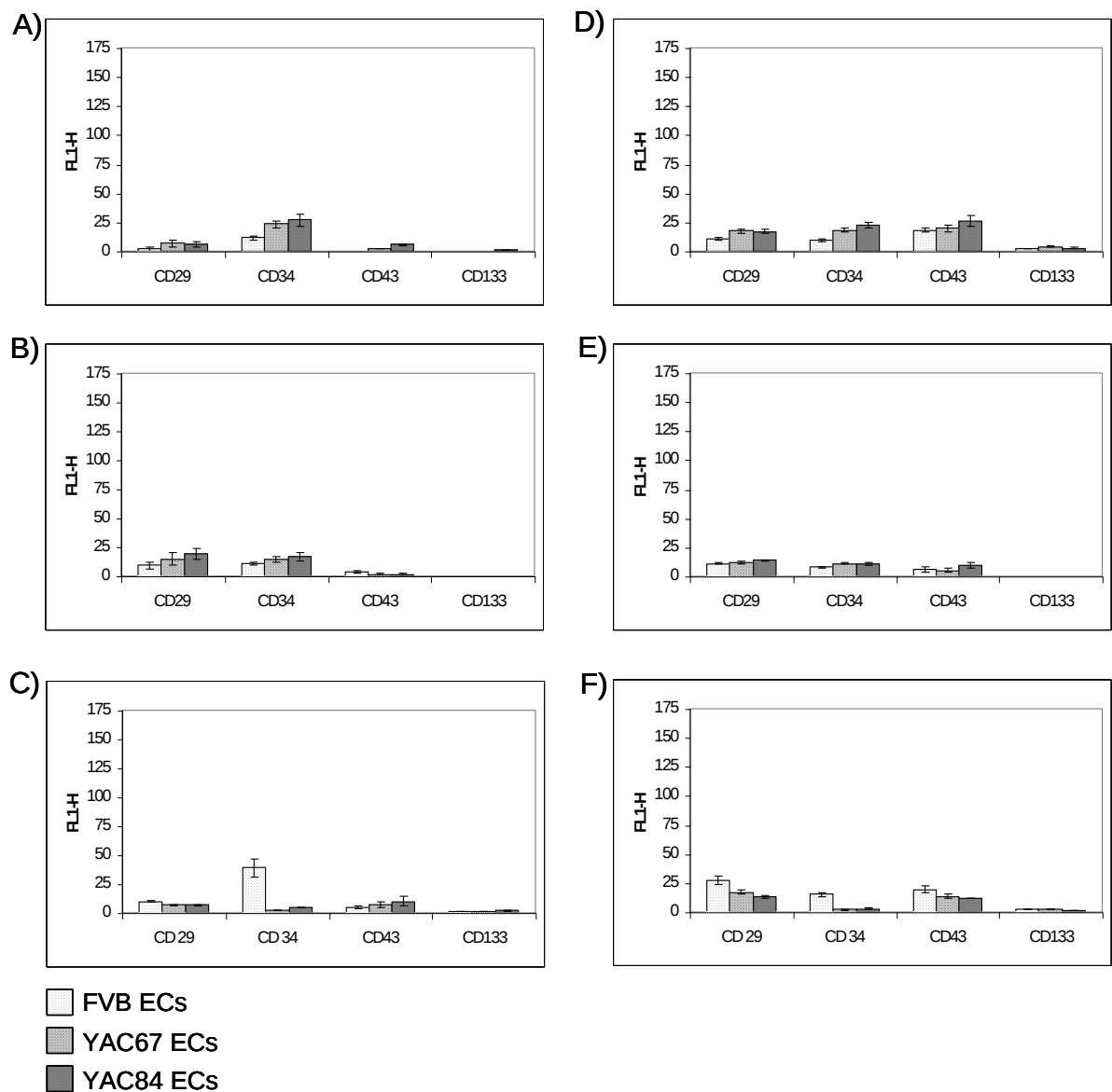


Figure 3.3. Expression of CD29, CD34, CD43 and CD133 molecules on ECs from transgenic and non-transgenic mice

Collagenase-collected, non-permeabilised cells from PLN (A), PP (B) and brain (C) as well as cells permeabilised from PLN (D), PP (E) and brain (F) were labeled with rat IgG2a anti-mouse CD29, rat IgG2a anti-mouse CD34 (RAM 34), rat IgG2a anti-mouse CD43, rat IgG1 anti-mouse CD133 and isotypic control further revealed by goat FITC-anti-rat immunoglobulin F(ab')₂. Green fluorescence intensity FL1-H (horizontal axis) represents delta value of the reactivity to the mAb and isotypic control. Graphs represent the mean of 4 experiments analysed by flow cytometry.

ECs isolated from transgenic mice revealed different phenotype as compared to cells from non-transgenic mice. The results presented express the mean value of four separate experiments.

As shown in figures 3.2 and 3.3, the labeling with anti-CD29 immunoglobulin reveals an opposite pattern of expression among the EC lines from primary lymphoid organs as compared to the secondary lymphoid organ and non directly related organ as the brain. The peripheral lymph node derived ECs (mPLNMEC) displayed an increased but weak expression in both YAC67 and YAC 84 versus FVB (Fig. 3.3 A). This phenomenon is similar although more clear in the case of Peyer's patches derived cells (Fig. 3.3 B).

Remarkably, this modulation is reversed considering the expression pattern of the CD29 among the brain-derived ECs (Fig. 3.3 C). Figure 3.2 indicates that the primary lymphoid organs-derived ECs (A and B) do not differ significantly in term of CD29 expression on their surface.

When considering the intracellular detection of this molecule, it appears that the same tendencies of the protein expression can be observed for mPLNMEC (Fig. 3.3 D), mPPMEC (Fig. 3.3 E) and mBrMEC (Fig. 3.3 F) as for the intact cells, whereas it is clearly differently modulated in the mBMMEC (Fig. 3.2 C) and mThMEC cell lines (Fig. 3.2 D).

The CD43 antigen expression pattern has been revealed mainly on permeabilized ECs. Intact endothelial cells have shown no or almost no CD43 expression (Fig. 3.2 and Fig. 3.3). Consequently, the following observations concern the experiments performed on permeabilized cells. Both ECs from transgenic mice, isolated from bone marrow have shown slightly lower CD43 as compared to control FVB cells (Fig. 3.2 C). Thymic ECs from YAC67 mice revealed lower CD43 expression than control FVB cells and not changed expression in YAC84 ECs (Fig. 3.2 D). In ECs from YAC84 PLN an increased CD34 expression, and no changes in YAC67 cells could be observed (Fig. 3.3 D).

Endothelial cells from Peyer's patches were characterized as expressing slightly higher level of CD43 in YAC84 and as compared to FVB and YAC67 cells (Fig. 3.3 E). Both ECs from brain of transgenic animals presented decreased CD43 expression when compared to FVB cells (Fig. 3.4 F).

Labeling with anti-CD133 antibody, detecting early hematopoietic and endothelial stem cell antigen, was performed. The expression of CD133 antigen was not found on any of the cell lines, either on the intact or permeabilized cells (Fig. 3.2 and Fig. 3.3).

3.4 ADHESIVE PROPERTIES OF ORGANOSPECIFIC ECS FROM TRANSGENIC ANIMALS TOWARD T AND B LYMPHOMA CELLS COMPARED TO FVB ENDOTHELIAL CELLS

Because one of the most significant role of endothelial cells is their ability to control homing and extravasation of circulating cells their adhesive properties were investigated. The characterization of the adhesion molecules protein level in transgenic versus control endothelial cells has revealed important changes in the expression of CD34 and, to lower extent, of CD29 and CD43. Those changes have appeared in all investigated transgenic animal-derived cells, namely cells isolated from bone marrow, thymus, peripheral lymph nodes, Peyer's patches and from brain. Due to these alterations it was necessary to verify how the changes in adhesion molecule levels could influence endothelial cells functions.

Adhesive interactions of transgenic and non-transgenic animal-derived ECs toward lymphocytic cell lines: EL4 (T lymphoid), EL4.IL-2 (activated T lymphoid) and RAW 8.1 (B lymphoid), were compared. The adhesion was performed in static conditions as described in Materials and Methods and the adhesion was quantified by flow cytometry which allows to identify the endothelial cells from the lymphocytes by the scattered light properties as well as the fluorescence emitted by the PKH26GL fluorochrome labeled lymphocytes (Fig. 3.4 and Fig. 3.5) as opposed to the non labeled ECs.

T cell lines adhesion profiles: Data presented in figures 3.4 and 3.5 show that both non-activated (EL4) and activated (EL4.IL-2) T lymphoid cells displayed decreased adhesion abilities towards all investigated endothelial cells from transgenic animals.

EL4.IL-2 T lymphoid cells, which secrete IL-2 molecule, adhered to endothelial cells with much higher efficiency (up to 3 lymphocytes per one EC) than non-activated lymphocytes (up to 1 lymphocyte per one EC).

All investigated endothelial cells (except mPPMEC YAC67 – Fig. 3.5 B) have decreased adhesion abilities of EL4 and EL4.IL-2 lymphocytes towards transgenic endothelial cells as compared to the control cells.

B cell lines adhesion profiles: Adhesion properties of B lymphocytes as tested with the help of the B lymphoid line RAW8.1 (Fig. 3.4, 3.5) showed strong differences in their adhesion properties towards ECs from FVB as compared to ECs from transgenic animals. This happened to be the case in the majority of organs the ECs derived from.

As an exception BM ECs (Fig. 3.4 A) have shown no significant diminution in adhesion towards YAC67 and YAC84 - ECs as compared to control FVB cells. In the case of thymus (Fig. 3.4 B), peripheral lymph nodes (Fig. 3.5 A) and brain (Fig. 3.5 C) the adhesion abilities gave the pattern FVB> YAC67> YAC84. In the case of Peyer's patches (Fig. 3.5 B) the amount of adhered B lymphocytes was slightly higher in the case of mPPMEC YAC67 and lower in mPPMEC YAC84 as compared to mPPMEC FVB cells.

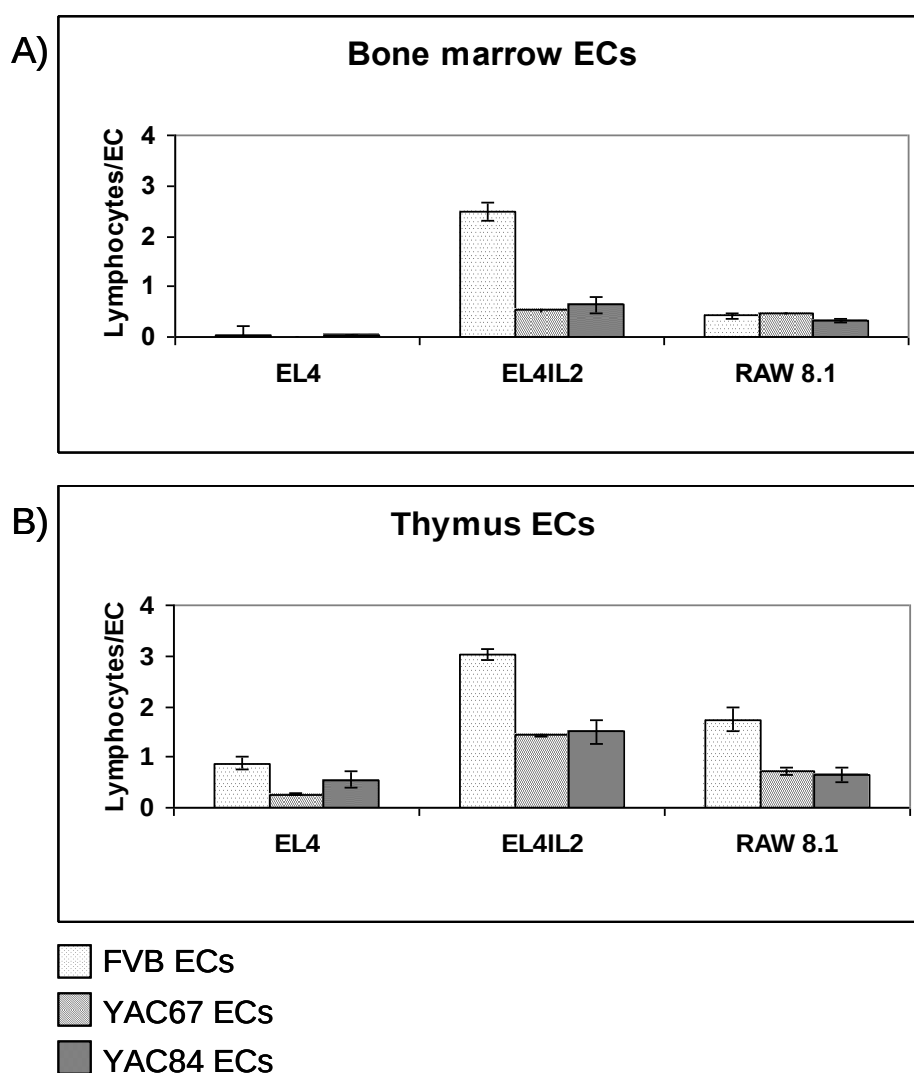


Figure 3.4. Adhesion properties of mouse endothelial cells from transgenic compared to control mice

The adhesion properties of bone marrow (A) and thymus (B) ECs were checked toward lymphoid cell lines. The proportion was: 5 labeled EL4, EL4.IL-2 and RAW 8.1 lymphocytes to 1 EC. The numbers of lymphocytes that adhered to EC was checked by flow cytometry. Graphs represent the mean of 3 experiments, data express the final ratio of lymphoid cells to 1 EC.

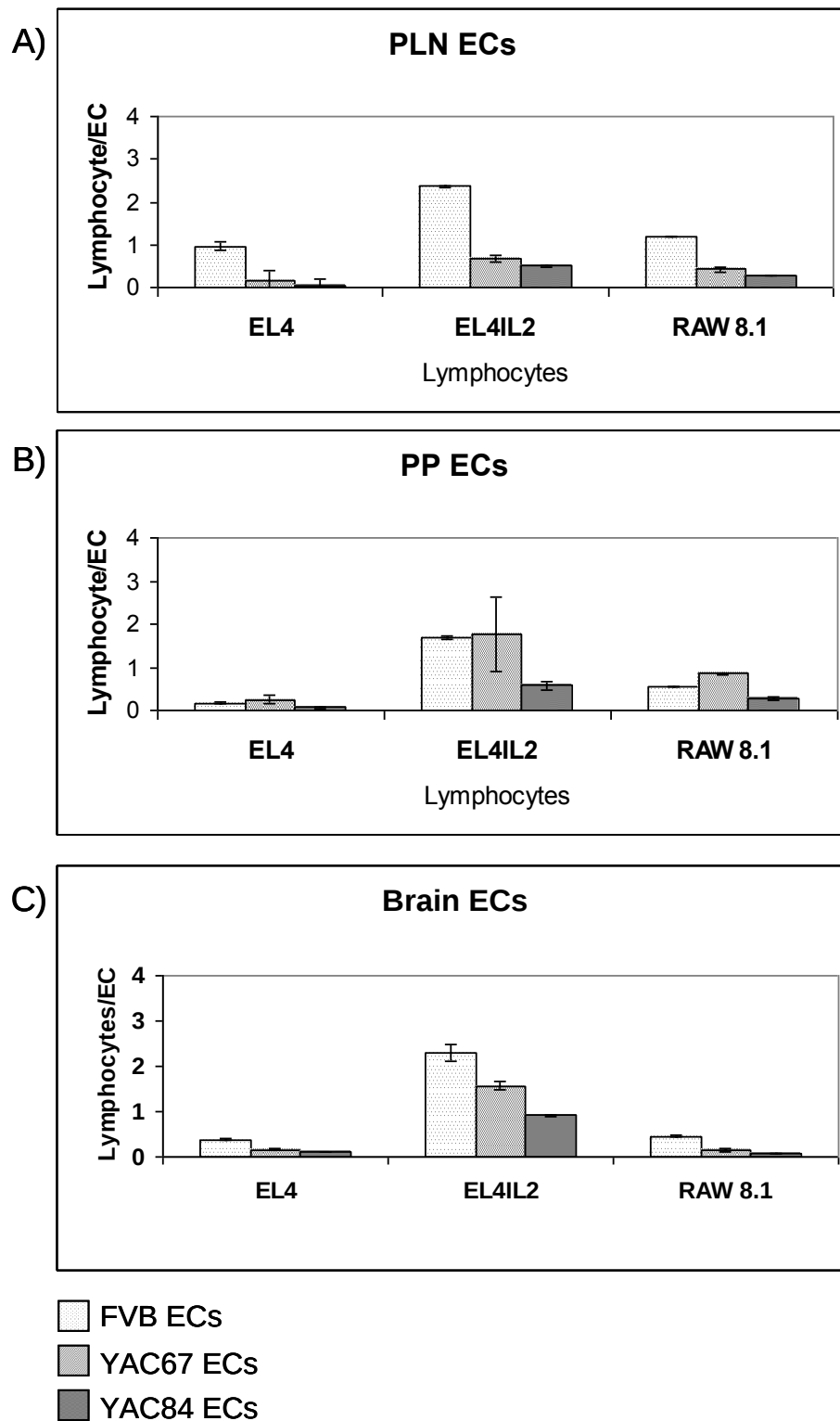


Figure 3.5. Adhesion properties of mouse endothelial cells from transgenic as compared to control mice

The adhesion properties of PLN (A), PP (B) and brain (C) ECs were checked towards lymphoid cell lines. The proportion was: 5 labeled EL4, EL4.IL-2 and RAW 8.1 lymphocytes to 1 EC. The numbers of lymphocytes that adhered to EC was checked by flow cytometry. Graphs represent the mean of 3 experiments, data express the final ratio of lymphoid cells to 1 EC.

3.5 ADHESIVE PROPERTIES OF ORGANOSPECIFIC ECS FROM TRANSGENIC ANIMALS TOWARD LYMPHOCYTES ISOLATED FROM FVB MICE

The above presented adhesion capacities of endothelial cells from transgenic mice towards EL4, EL4.IL-2 and RAW 8.1 lines were next compared to the adhesion process of isolated T and B lymphocytes.

The potential influence of *GIRK2* gene overexpression due to the presence of additional copies, on the adhesion properties of ECs was investigated.

For this purpose, thymus- and spleen-isolated lymphocytes were overlaid onto transgenic and control endothelial cell cultures (Fig. 3.6, 3.7). ECs from transgenic animals have shown changed adhesion profiles as compared to control FVB-derived cells.

Thymocytes adhesion pattern: thymocytes adhered better to transgenic than to control BM ECs cells (Fig. 3.6 A), in parallel with the presence of *GIRK2* gene copies. This can be significant for the localizations of T lymphocytes and their arrest. Thymocytes adhesion to ECs from thymus (Fig. 3.6 B), PLN, PP and brain (Fig. 3.7 A, B, C) was very weak as it could be expected for normal thymocytes (Picker, Terstappen et al. 1990; Mebius, Streeter et al. 1996).

Spleen cells adhesion pattern: Lymphocytes isolated from spleen adhered to transgenic and control mBMMEC as follows: FVB < YAC67 < YAC84 (Fig. 3.6 A). On the contrary, the adhesion pattern towards mThMEC gave the opposite results: FVB > YAC67 > YAC84 (Fig. 3.6 B).

PLN ECs from YAC67 mice show slightly weaker adhesion abilities comparing to control cells. There were no statistically significant differences between mPLNMEC FVB and mPLNMEC YAC84 cells (Fig. 3.7 A). All PP ECs (from transgenic and non-transgenic mice) have shown comparable adhesion capacities (Fig. 3.7 B). Among brain ECs the line YAC67 with 2 additional *GIRK2* copies displayed slightly stronger adhesion ability than the YAC84 line and the normal counterpart (Fig. 3.7 C).

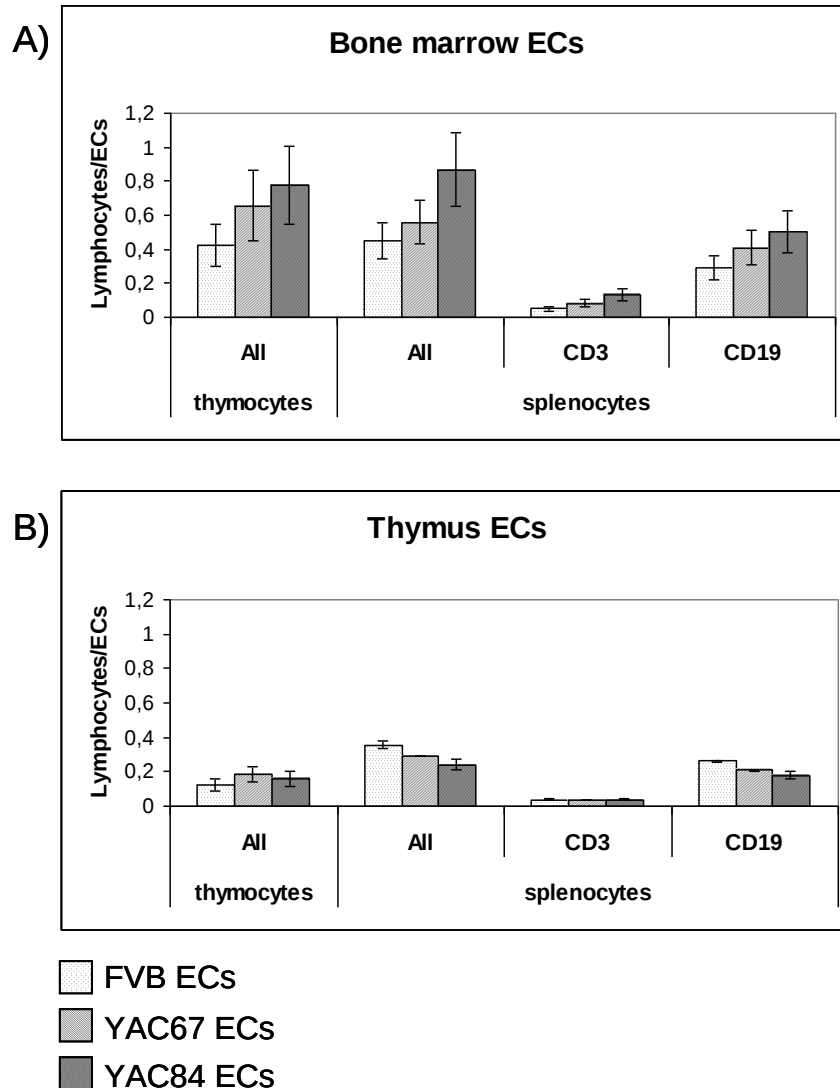


Figure 3.6. Adhesion properties of mouse endothelial cells from transgenic as compared to control mice

The adhesion properties of bone marrow (A) and thymus (B) ECs were checked towards lymphocytes isolated from thymus and spleen. The proportion was: 5 lymphocytes to 1 EC. The numbers of lymphocytes that adhered to EC was checked by flow cytometry. Graphs represent the mean of 3 experiments, data express the final ratio of lymphoid cells to 1 EC.

All –all adhered lymphocytes; CD3 –CD3 positive lymphocytes; CD19 –CD19 positive lymphocytes.

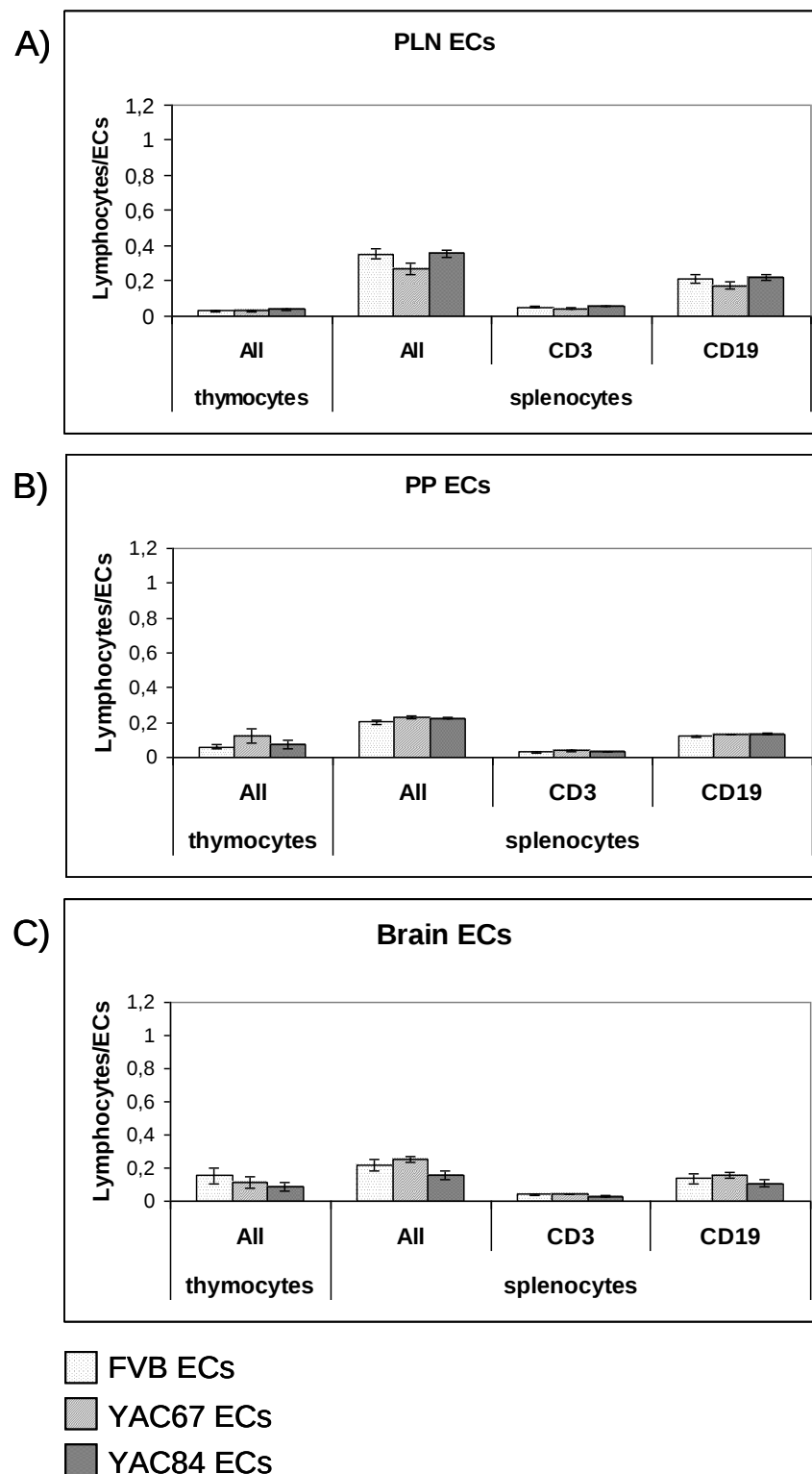


Figure 3.7. Adhesion properties of mouse endothelial cells from transgenic as compared to control mice

The adhesion properties of PLN (A), PP (B) and brain (C) ECs were checked towards lymphocytes isolated from thymus and spleen. The proportion was: 5 lymphocytes to 1 EC. The numbers of lymphocytes that adhered to EC was checked by flow cytometry. Graphs represent the mean of 3 experiments, data express the final ratio of lymphoid cells to 1 EC.

All –all adhered lymphocytes; CD3 –CD3 positive lymphocytes; CD19 –CD19 positive lymphocytes.

3.6 COMPARISON OF THE ANGIOGENIC PROPERTIES OF ECS FROM TRANSGENIC MICE WITH ADDITIONAL COPIES OF *GIRK2* GENE

One of the main features of the endothelium is the ability of new vessels formation. *In vivo*, angiogenesis is regulated by pro- and anti-angiogenic molecules secreted by endothelial cells, monocytes/macrophages, fibroblasts (Moldovan and Moldovan 2005; Karamysheva 2008).

Angiogenic properties of transgenic ECs were verified *in vitro* using MatrigelTM. Being a pseudo matrix, the MatrigelTM contains many types of molecules among which are factors that regulate angiogenesis. As such it has become the most frequently used substance for angiogenesis process investigation. Endothelial cells cultured on MatrigelTM migrate, elongate and create a net mimicking the vessels. To investigate the ability of YAC ECs to create pseudovessels the cells were cultured on MatrigelTM for 24 hr.

It occurred that all transgenic endothelial cells show strong abnormalities in their angiogenic behavior as compared to normal non-transgenic mice-derived ECs (Fig. 3.8, 3.9, 3.10).

In the case of bone marrow ECs (Fig. 3.8 A) the differences were observed only after 24 hr. Indeed no differences were visible between transgenic and non-transgenic mice-derived cells after 5 and 12 hr. In those BM lines angiogenesis proceeded normally, but after 24 hr mBMMEC YAC84 was reversed completely and only brushes-like structures were present.

The ECs of thymus origin (Fig. 3.8 B): mThMEC FVB, mThMEC YAC67 and mThMEC YAC84 started to display the differences in pseudovessels formation after 5 hr of culture in MatrigelTM. After 12 hr of culture non-transgenic mThMEC FVB were organized into pseudotubes, while mThMEC YAC67 hardly started to rearrange and mThMEC YAC84 showed no angiogenic behavior, forming after 24 hr a brush-like structure. No vessel formation was detectable.

ECs isolated from peripheral lymph nodes (Fig. 3.9 A) and Peyer's patches (Fig. 3.9 B) have shown an opposite phenomenon. In the case of mPLNMEC FVB the process of angiogenesis was not visible after 5 hr of culture on MatrigelTM. They did not develop more advanced net until 12 hr of culture. Only after 24 hr a progress in angiogenesis process was noticeable. On the contrary both transgenic lines mPLNMEC YAC67 and mPLNMEC YAC84 have shown more advanced angiogenesis than control cells already after 5 hr of culture. The angiogenesis process was progressing after 12 hr. After 24 hr well established pseudovessels were visible.

mPPMEC FVB (Fig. 3.9 B) line reacted similarly to mPLNMEC FVB. The progress in angiogenesis process was very slow and after 12 hr the cells were barely elongated, some of them touch each other and created fragments of the net. A lot of cells were not elongated and did not react. After 24 hr the net was not more developed indicating that the angiogenesis was not progressing. Both transgenic lines from Peyer's patches showed stronger angiogenic abilities compared to the control line. This was noticeable already after 5 hr of cells cultured on MatrigelTM. Angiogenesis process was progressing during the 24 hr of observation. Both transgenic lines created pseudovessels. In the case of ECs from Peyer's patches the progress of angiogenesis process was the fastest in YAC84 line and the slowest in FVB control line which showed almost no angiogenesis.

Brain ECs from transgenic mice (Fig. 3.10) also displayed profound differences in angiogenic behavior, as compared to their non-transgenic partners. For mBrMEC FVB cells, pseudovessels formation started to be noticeable as soon as after 5 hr, the tube-like structures were clearly visible. mBrMEC YAC67 and YAC 84 lines did not undergo angiogenesis, only brushes-like structures were present.

The above presented results have pointed out disturbed angiogenic properties in transgenic mice-derived endothelial cells isolated from bone marrow, thymus and brain. On the contrary, transgenic mice-derived endothelial cells isolated from peripheral lymph nodes and Peyer's patches developed more efficiently pseudovessels as compared to the control FVB cells.

Because the adhesion properties of the endothelial cells derived from transgenic mice were largely disturbed the hypothesis according to which the endothelial cells could influence the journey, selection and finally homing of lymphocytes in a different manner in the trisomic model than in the normal individuals was supported. The angiogenesis kinetics and efficiency was also greatly affected thus confirming a strong involvement of the endothelial cells in the immune response of the *GIRK2* transgenic animal models.

The complexity of the phenomenon prompted us to address the question whether a general feature of the expression pattern of the main molecules involved in the key steps of the lymphocyte homing can be evidenced. A special attention was focused on adhesion molecules, chemokines and their receptors as well as ECs growth factors.

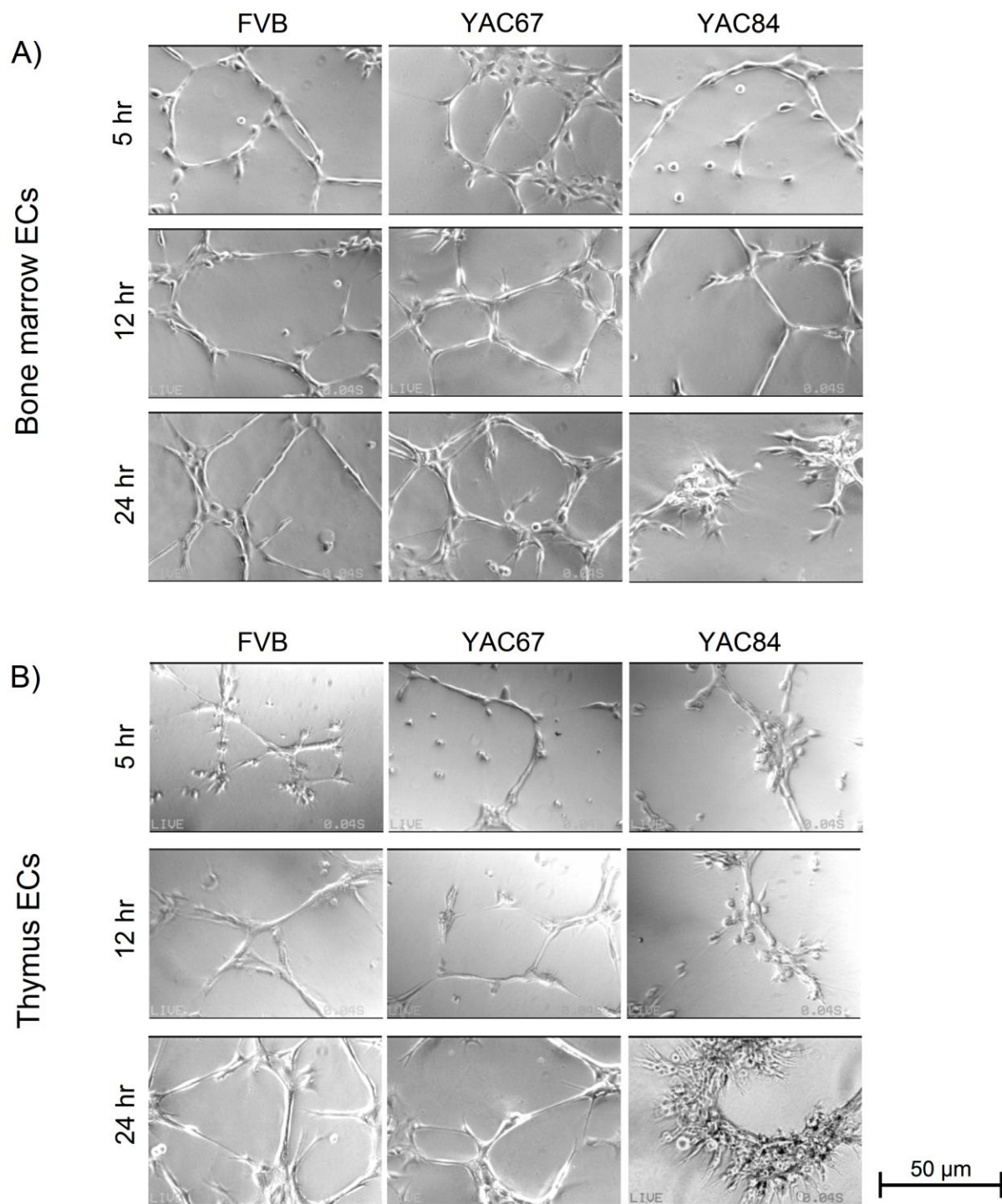


Figure 3.8. Pseudovessels formation properties of FVB, YAC67 and YAC84 ECs isolated from bone marrow (A) and thymus (B)

Cells were seeded on Matrigel coated wells (Becton Dickinson). The observations were conducted at 5, 12 and 24 hr after seeding (see also Materials and Methods) by phase contrast microscopy (Zeiss, Axiovert 200M).

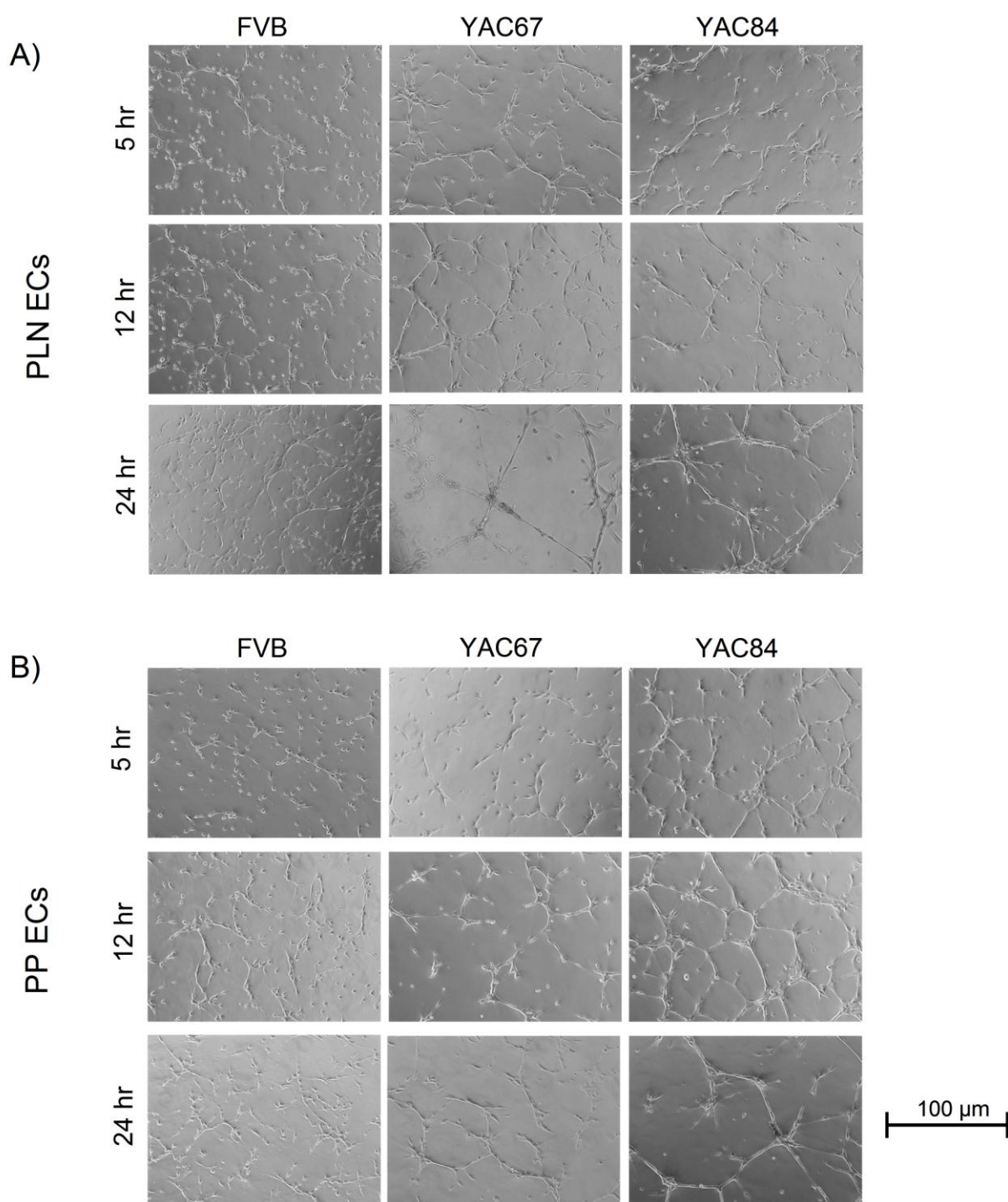


Figure 3.9. Pseudovessels formation properties of FVB, YAC67 and YAC84 ECs isolated from PLN (A) and PP (B)

Cells were seeded on Matrigel coated wells (Becton Dickinson). The observations were conducted at 5, 12 and 24 hr after seeding (see also Materials and Methods) by phase contrast microscopy (Zeiss, Axiovert 200M).

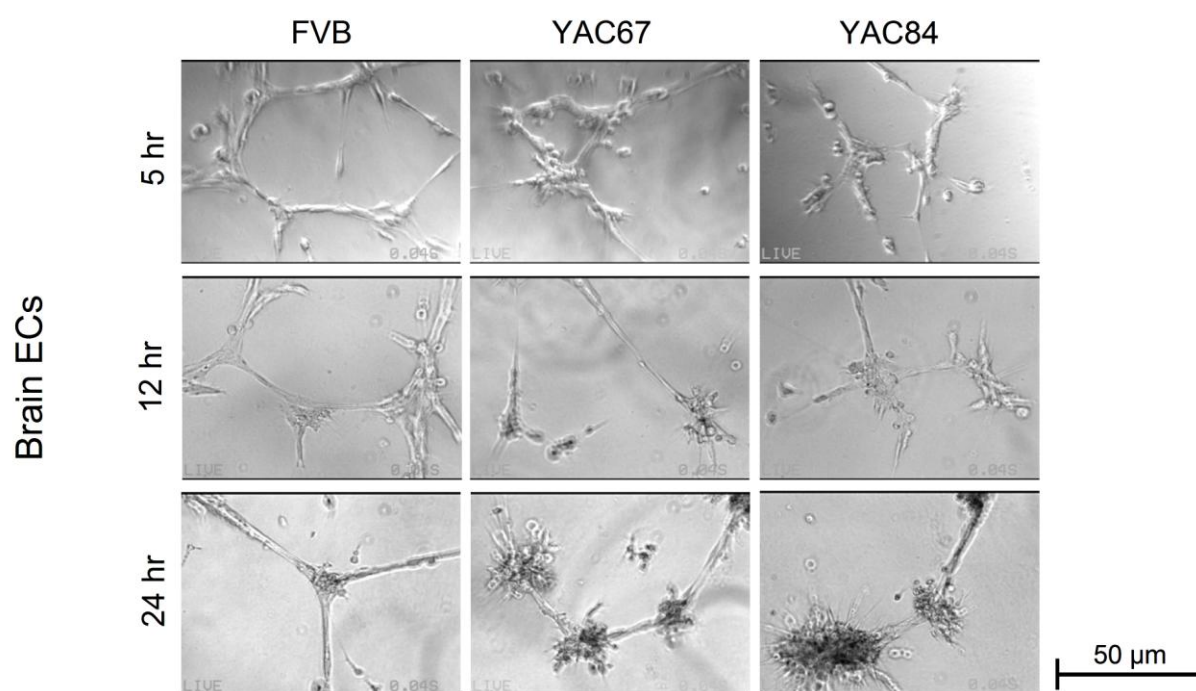


Figure 3.10. Pseudovessels formation properties of FVB, YAC67 and YAC84 ECs isolated from brain

Cells were seeded on Matrigel coated wells (Becton Dickinson). The observations were conducted at 5, 12 and 24 hr after seeding (see also Materials and Methods) by phase contrast microscopy (Zeiss, Axiovert 200M).

3.7 GENE EXPRESSION ASSESSMENT BY cDNA MICROARRAYS FOR THE WHOLE MURINE GENOME

The above presented results have shown changes in all investigated transgenic endothelial cells as compared to control FVB cells. These changes concern many aspects of endothelial cells functionality like adhesion and angiogenesis. Moreover those changes are present in cells isolated from different organs: bone marrow, thymus, peripheral lymph nodes, Peyer's patches and brain.

To be able to answer the question concerning the changes in expression of which particular genes are responsible for all known alterations a gene array was undertaken. This allowed comparing of all the EC lines obtained, especially in terms of molecules involved in the lymphocytes homing, their exportation pathways, mobilization during the inflammation as well as angiogenesis process. For the analysis several genes characteristic for those processes were chosen.

They were grouped into three categories:

- adhesion molecules,
- chemokines and receptors for chemokines,
- Vegfs and receptors for Vegfs.

Their expression values were presented as a fold range between the expression values of genes from transgenic mice-derived ECs and expression values of genes from FVB cells.

In the adhesion molecules group the expression of the following genes was investigated: E-selectin, P-selectin, Madcam-1, Pecam-1, Vcam-1, Icam-1, Icam-2, Icam-4, CD29, CD34, CD43, CD44, CD133. Bone marrow mRNA (Fig. 3.11 A) isolated from transgenic mice-derived endothelial cells has shown changes in the maximal range of 2.5 as compared to control ECs. Expression of Vcam-1 was twice higher in YAC67 and was not changed in YAC84 ECs. CD133 expression increase took place in YAC84 cells. The expression of Pecam-1 molecule was unchanged in the case of YAC67 cells and twofold higher in the case of YAC84 cells as compared to FVB cells. Some genes expression decrease could be observed too. The most significant expression decrease (2.4 times) was visible for Icam-4 from YAC84 ECs as compared to FVB cells.

In transgenic mice-derived endothelial cells isolated from thymus (Fig. 3.11 B) the most significant changes concern E-selectin and CD34 molecules. The expression of E-selectin has been decreased 3 times in YAC67 cells and 16 times (ratio YAC67/FVB = 0.06) in YAC84 cells. CD34 expression is 10 times higher in both YAC67 and YAC84 cells as compared to FVB control cells. CD133 expression is 1.5 times higher in YAC67 cells and 4 times higher in YAC84 cells.

YAC67 ECs isolated from PLNs (Fig. 3.12 A) display a slightly decreased (1.7 times) E-selectin expression, while YAC84 ECs have a 4 times increased mRNA level as compared to the FVB cells. Another molecule with changed expression is Pecam-1 with an almost 8 times higher mRNA level in YAC67 cells but slightly higher (twice) in YAC84 cells. Vcam-1 molecule had a twice lower expression in YAC84 cells. Icam-1 expression was 8.3 times lower in YAC67 and 3.3 times lower in YAC84 endothelial cells. Icam-2 displayed very strong changes in its expression level in the transgenic animal-derived ECs. These variations were the most significant among all adhesion molecules investigated. Indeed, Icam-2 expression was 61x higher in YAC67 cells and 76.7x higher in YAC84 cells than in normal PLN ECs. Icam-4 expression was increased by a factor of 2.6 but only in YAC67 cells. A significant change concerns the expression of CD43 molecule. Its expression has been increased both in YAC67 cells (4.5 times) as well as in YAC84 cells (5.4 times).

The expression of CD133 gene was 2.1 times decreased in YAC67 cells in comparison to FVB endothelial cells.

In endothelial cells isolated from Peyer's patches (Fig. 3.12 B), the adhesion molecules pattern of expression indicates that the main changes concern Pecam-1, Icam-1 and, to a lesser extent, Vcam-1, CD34 and CD43. The expression of Pecam-1 was decreased 5.8 times but only in YAC84 cells; the expression of Vcam-1 was decreased 3 times in YAC67 cells and display a slightly increased expression in YAC84 cells. The expression of Icam-1 was increased 6.2 times in YAC67 cells only. In the case of CD34 molecule the mRNA level was increased 2.2 times in YAC67; CD43 expression was 3.4 times increased in YAC84 cells.

The expression pattern of the adhesion molecules in endothelial cells from brain (Fig. 3.13) was characterized by a 1.9 times decrease in E-selectin, and 2.4 times decrease of the Pecam-1 mRNA level in YAC67. Brain-derived ECs displayed an increased expression of Icam-1 (6 times in YAC67 and 2.5 times in YAC84) and Icam-4 (4.3 times in both YAC67 and YAC84). CD34 appears upregulated in the YAC84 transgenic mice brain derived ECs by 2.7 times.

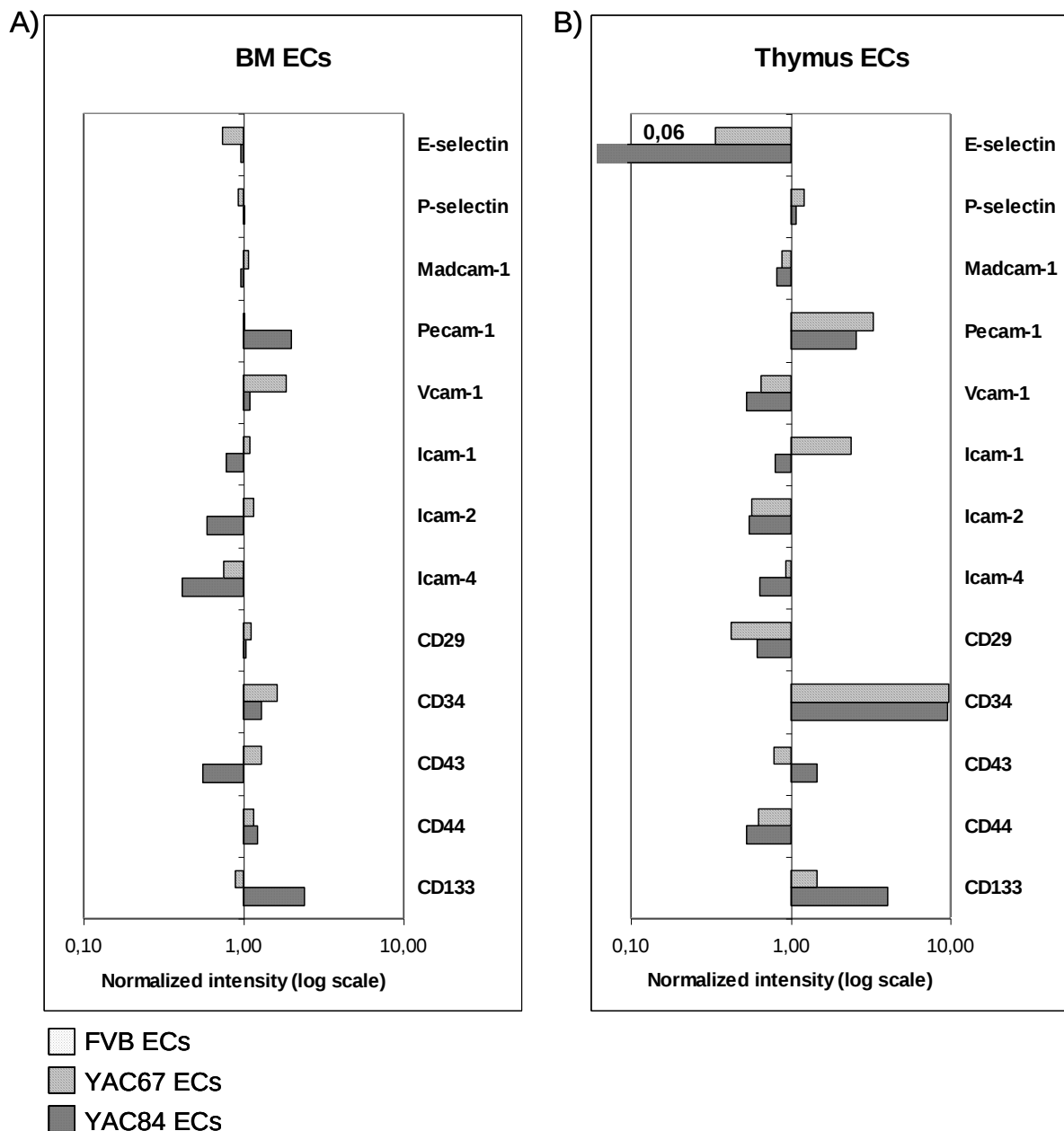


Figure 3.11. Adhesion molecules gene expression profile of mouse endothelial cells from transgenic as compared to control mice

Adhesion molecules gene expression profile of bone marrow (A) and thymus (B) ECs coming from transgenic mice YAC67, YAC84 in comparison to control FVB ECs was investigated by cDNA microarray analysis. Samples were tested towards the reference sample which was the mix of RNA isolated from each cell culture. Graphs show the fold range of transgenic mice-derived ECs adhesion molecules expression level (as the optical density – horizontal axis) towards the adhesion molecules expressed by FVB ECs as means for two independent experiments. Below 1 are placed the genes with expression level lower than FVB sample. Above 1 are placed the genes with expression level higher than reference sample.

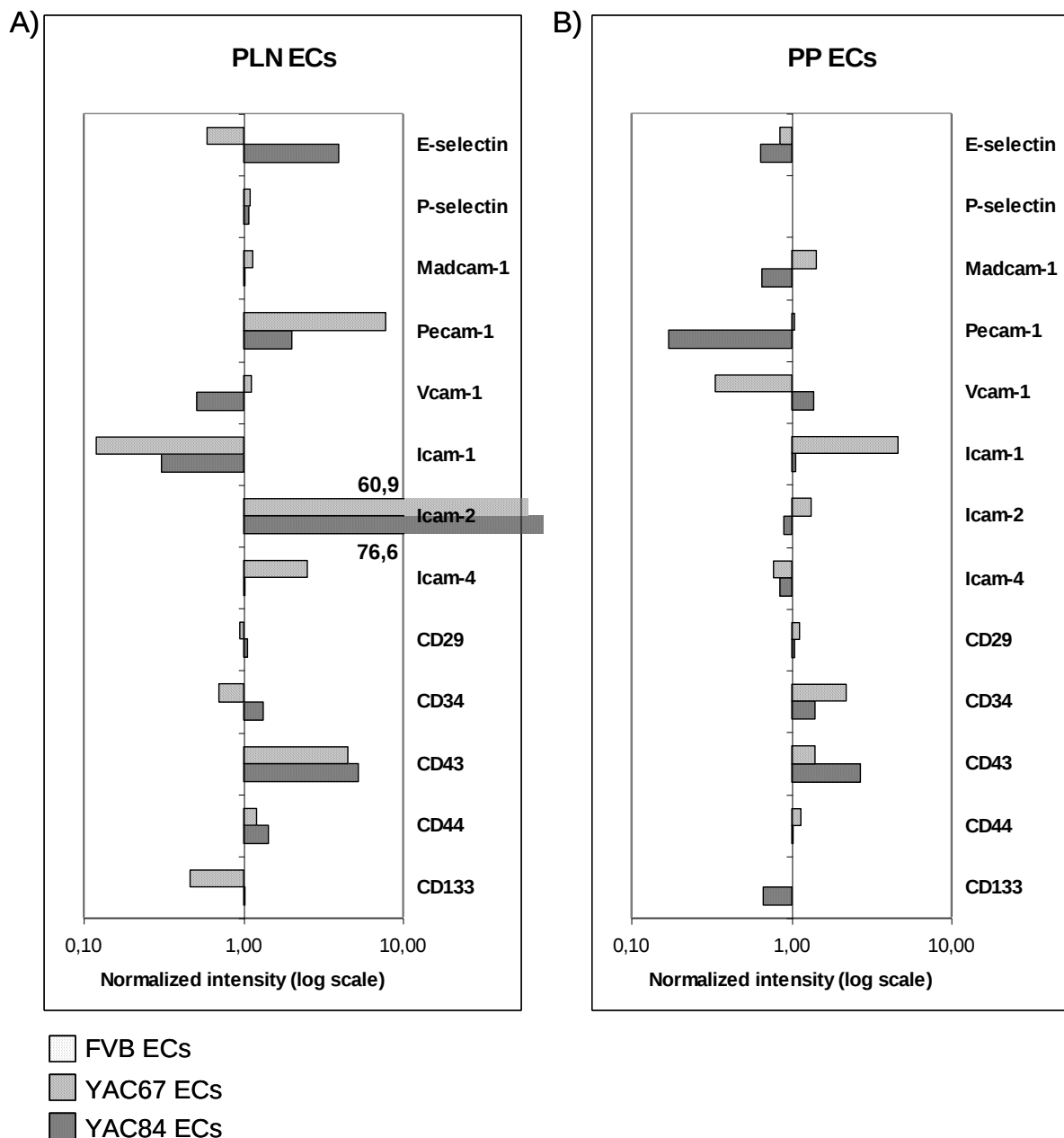


Figure 3.12. Adhesion molecules gene expression profile of mouse endothelial cells from transgenic as compared to control mice

Adhesion molecules gene expression profile of PLN (A) and PP (B) ECs coming from transgenic mice YAC67, YAC84 in comparison to control FVB ECs was investigated by cDNA microarray analysis. Samples were tested towards the reference sample which was the mix of RNA isolated from each cell culture. Graphs show the fold range of transgenic mice-derived ECs adhesion molecules expression level (as the optical density – horizontal axis) towards the adhesion molecules expressed by FVB ECs as means for two independent experiments. Below 1 are placed the genes with expression level lower than FVB sample. Above 1 are placed the genes with expression level higher than reference sample.

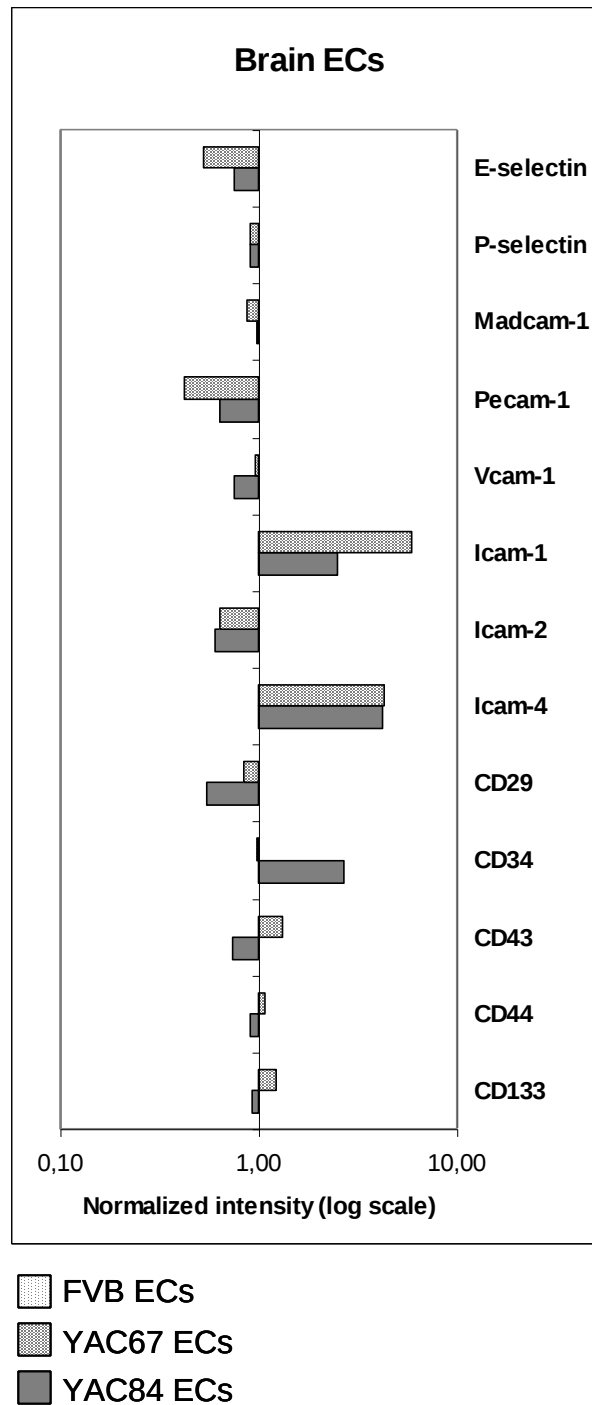


Figure 3.13. Adhesion molecules gene expression profile of mouse endothelial cells from transgenic as compared to control mice

Adhesion molecules gene expression profile of brain ECs coming from transgenic mice YAC67, YAC84 in comparison to control FVB ECs was investigated by cDNA microarray analysis. Samples were tested towards the reference sample which was the mix of RNA isolated from each cell culture. Graphs show the fold range of transgenic mice-derived ECs adhesion molecules expression level (as the optical density – horizontal axis) towards the adhesion molecules expressed by FVB ECs as means for two independent experiments. Below 1 are placed the genes with expression level lower than FVB sample. Above 1 are placed the genes with expression level higher than reference sample.

The next group of genes that was investigated concerns chemokines and receptors for chemokines. This group includes Cxcl1, Cxcl2, Cxcl9, Cxcl10, Cxcl12, Ccl2, Ccl3, Ccl4, Ccl5, and the following receptors Cxcr2, Cxcr3, Cxcr4, Ccr2, Ccr3, Ccr5.

In ECs isolated from bone marrow (Fig. 3.14 A) the most significant changes in genes expression concerned Cxcl12 and Ccl5. The ratio YAC67/FVB and YAC84/FVB of Cxcl12 expression equaled 0.48 and 0.27 respectively, what resulted in the Cxcl12 expression pattern FVB>YAC67>YAC84. The ratio of Ccl5 equaled 5.13 and 1.32 (YAC67/FVB and YAC84/FVB respectively) what gave 5 times higher Ccl5 expression in YAC67 cells and slightly increased expression in YAC84 endothelial cells as compared to control FVB cells.

In the case of ECs isolated from the thymus of transgenic mice (Fig. 3.14 B) significant expression changes were visible in several genes: Cxcl1, Cxcl10, Cxcl12, Ccl5 and Cxcr4. Ratio YAC67/FVB for Cxcl1 equaled 4.46. In YAC84 cells this gene had twice weaker expression as compared to FVB cells. Cxcl10 had 1.56 times higher expression in YAC67 cells and almost 16 times higher in YAC84 cells. Both transgenic mice-derived thymic endothelial cell lines have shown decreased Cxcl12 expression (6 and 5 times in YAC67 and YAC84 cells respectively) and increased Ccl5 expression (4 and 8.3 times in YAC67 and YAC84 cells respectively). Cxcr4 gene is differently modulated in YAC67, where it is increased (YAC67/FVB ratio of expression equaled 3.6) as opposed to YAC84 where it is considerably lowered (ratio YAC84/FVB = 0.02).

Transgenic mice-derived endothelial cells isolated from PLNs (Fig. 3.15 A) displayed significant changes in the gene expression concerning: Cxcr2 gene (ratio YAC67/FVB = 2.5; ratio YAC84/FVB = 0.3) and Ccr2 (ratio YAC67/FVB = 7.76; ratio YAC84/FVB = 0.6). The expression of Ccl4 gene was considerably decreased in YAC84 cells (ratio YAC84/FVB = 0.01) but not changed in YAC67 cells. Minor expression changes were observed for the following genes: Cxcl1 (decreased in both transgenic lines), Cxcl2 (increased in YAC67 cells and slightly in YAC84), Cxcl9 (decreased mainly in YAC67 cells) and Cxcl10 (decreased mainly in YAC84 cells), Ccl2 (increased in both transgenic lines), Cxcr4 (increased in YAC84 cells), Ccr5 (decreased in YAC67 cells).

Transgenic mice-derived endothelial cells isolated from Peyer's patches (Fig. 3.15 B) displayed significantly changed expression of the following genes: Cxcl10, Cxcl12, Ccl2, Ccl5, Cxcr4, Ccr2 and Ccr3. The ratios of Cxcl10 gene expression are 3.1 and 1.9 for YAC67/FVB and YAC84/FVB respectively. The expression of Cxcl12 gene is 7 times increased in YAC67 endothelial cells and slightly decreased in YAC84 cells. Both YAC67

and YAC84 endothelial lines have 4.4 times and 4.6 times increased Ccl2 expression (YAC67 and YAC84 respectively). In the case of Ccl5 gene the expression is increased 6 times in YAC67 and 2.7 times in YAC84 cells. The expression of Cxcr4 gene is considerably lowered in YAC67 cells but in YAC84 cells the expression of this gene is increased 4.4 times. Ccr2 expression is increased in both transgenic mice-derived endothelial lines (5.2 times and 5.9 times in YAC67 and YAC84 respectively). Ccr3 expression is increased in YAC84 cells (2.6 times) and not changed in YAC67 cells.

In transgenic mice-derived endothelial cells isolated from brain (Fig. 3.16) the most significant changes were detected in Cxcl10, Ccl4, Ccl5 and Cxcr4 expressions. The expression of Cxcl10 is decreased 2.9 and 1.4 times in YAC67 and YAC84 endothelial cells respectively. The mRNA level of Ccl4 was decreased 1.9 times in YAC84 cells and almost not changed in YAC67 cells. Both cell lines have increased expression of Ccl5 gene (3.6 and 2.7 times in YAC67 and YAC84 cells respectively). Cxcr4 mRNA level is modulated by a ratio 0.57 (YAC67/FVB) and 0.37 (YAC84/FVB).

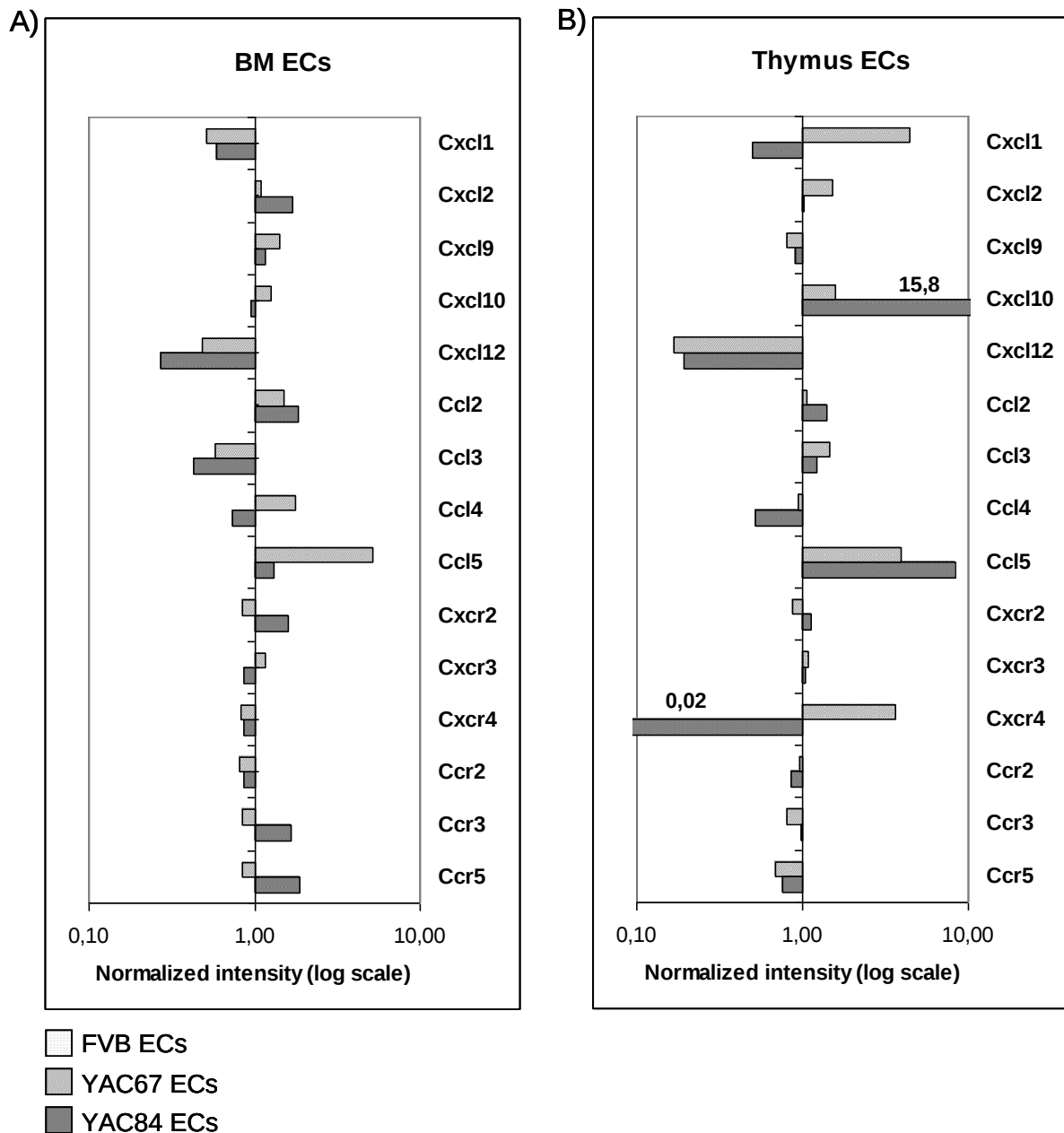


Figure 3.14. Chemokines and receptors for chemokines genes expression profile of mouse endothelial cells from transgenic as compared to control mice

Chemokines and receptors for chemokines genes expression profile of bone marrow (A) and thymus (B) ECs coming from transgenic mice YAC67, YAC84 in comparison to control FVB ECs was investigated by cDNA microarray analysis. Samples were tested towards the reference sample which was the mix of RNA isolated from each cell culture. Graphs show the fold range of transgenic mice-derived ECs adhesion molecules expression level (as the optical density – horizontal axis) towards the adhesion molecules expressed by FVB ECs as means for two independent experiments. Below 1 are placed the genes with expression level lower than FVB sample. Above 1 are placed the genes with expression level higher than reference sample.

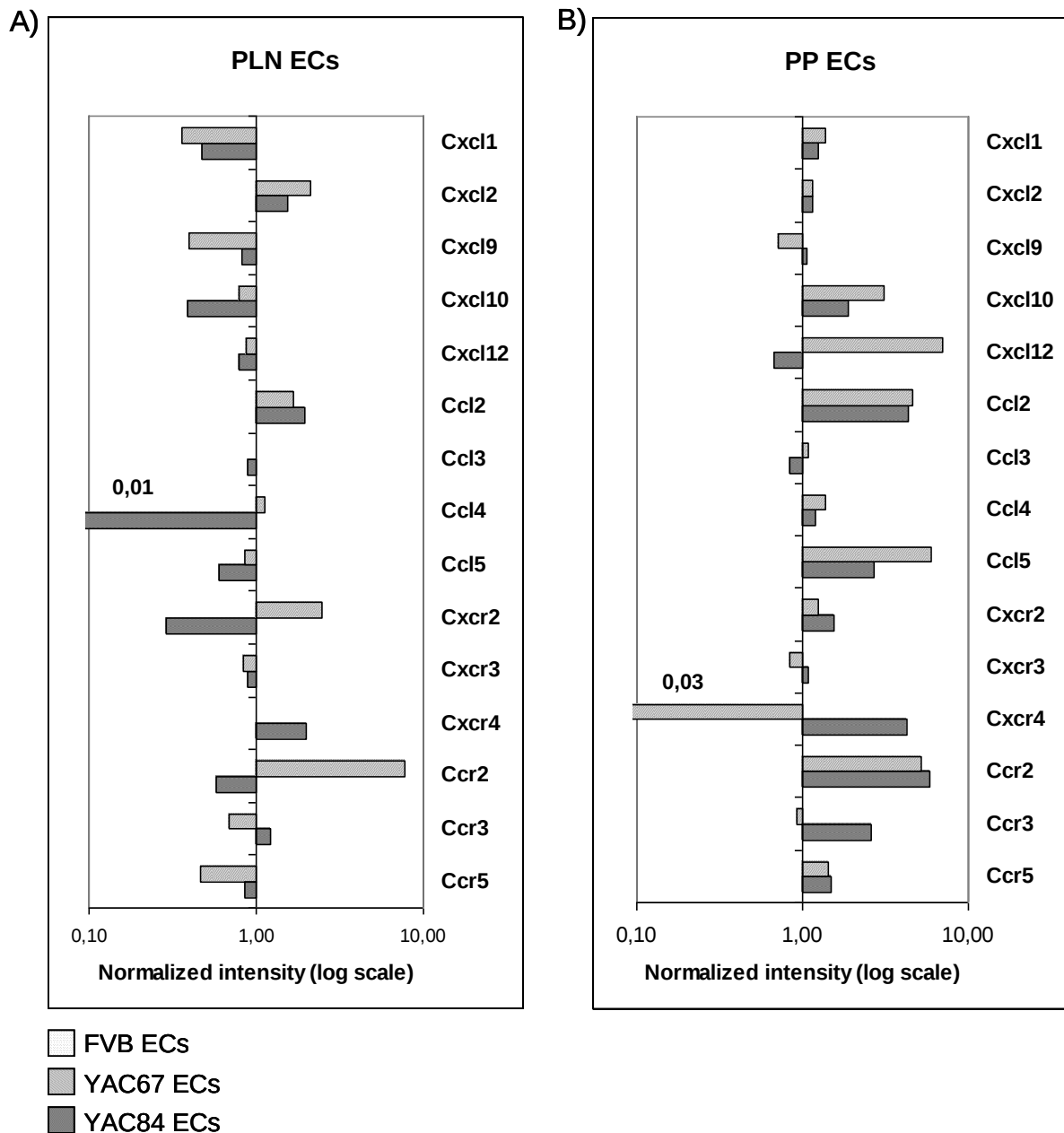


Figure 3.15. Chemokines and receptors for chemokines gene expression profile of mouse endothelial cells from transgenic as compared to control mice

Chemokines and receptors for chemokines gene expression profile of PLN (A) and PP (B) ECs coming from transgenic mice YAC67, YAC84 in comparison to control FVB ECs was investigated by cDNA microarray analysis. Samples were tested towards the reference sample which was the mix of RNA isolated from each cell culture. Graphs show the fold range of transgenic mice-derived ECs adhesion molecules expression level (as the optical density – horizontal axis) towards the adhesion molecules expressed by FVB ECs as means for two independent experiments. Below 1 are placed the genes with expression level lower than FVB sample. Above 1 are placed the genes with expression level higher than reference sample.

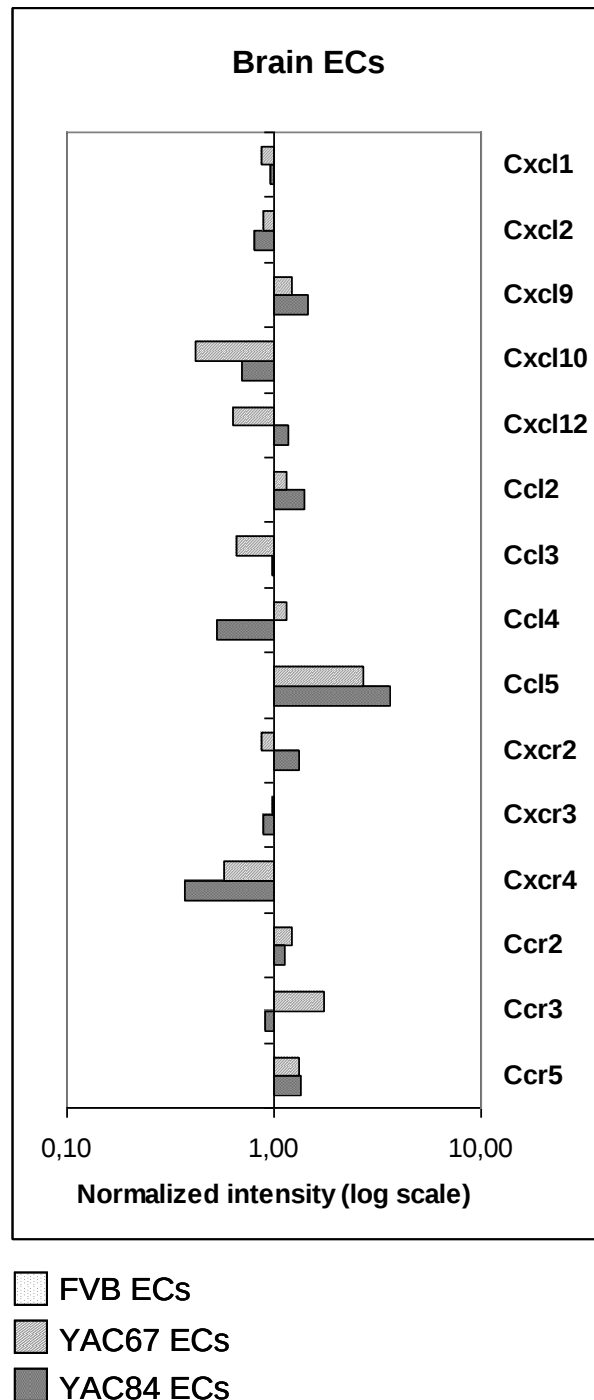


Figure 3.16. Chemokines and receptors for chemokines gene expression profile of mouse endothelial cells from transgenic as compared to control mice

Chemokines and receptors for chemokines gene expression profile of brain ECs coming from transgenic mice YAC67, YAC84 in comparison to control FVB ECs was investigated by cDNA microarray analysis. Samples were tested towards the reference sample which was the mix of RNA isolated from each cell culture. Graphs show the fold range of transgenic mice-derived ECs adhesion molecules expression level (as the optical density – horizontal axis) towards the adhesion molecules expressed by FVB ECs as means for two independent experiments. Below 1 are placed the genes with expression level lower than FVB sample. Above 1 are placed the genes with expression level higher than reference sample.

The vascular endothelial growth factors and their receptors are fundamental molecules that control angiogenesis, in term of endothelial cells growth and activation. They are mainly modulated by the balance between hypoxic versus normoxic conditions of the microenvironment (Ferrara 2004). Vegfs and Vegfs' receptors expression were checked. The expressions of the following genes were investigated: Vegf-A, Vegf-B, Vegf-C, Flt1 (Vegfr-1), Flk1 (Vegfr-2), Flt4 (Vegfr-3) (Fig. 3.17). In transgenic mice-derived bone marrow endothelial cells (Fig. 3.17 A) Vegf-A expression was decreased (4.4 times in YAC67 cells and 1.53 times in YAC84 cells). Vegf-C expression in these cells was decreased in YAC67 cells (1.86 times) and increased in YAC84 cells (2.56 times). In endothelial cells isolated from the thymus (Fig. 3.17 B) of YAC84 mice Vegf-A was increased 2.86 times as compared to control FVB cells. Both thymus derived transgenic lines displayed increased Vegf-B expression (2.17 and 2.1 times in YAC67 and YAC84 cells respectively). The expression of Vegf-C decreased in YAC67 cells 6.1 times and twice in YAC84 cells. In the case of endothelial cells isolated from PLNs (Fig. 3.17 C) the significant changes concern Vegf-A, the expression of which has been increased by 1.7 times in both transgenic lines; the expression of Vegf-B was decreased by 2.36 times in YAC67 cells and twice in YAC84 cells. The most significant change concern Flt4. In YAC67 cells the expression of this gene increased 7.8 times and in cells YAC84 13.3 times. In endothelial cells isolated from Peyer's patches (Fig. 3.17 D) significant changes were: 4 times lowering of Vegf-A expression in YAC67 cells and 1.6 times lowering in both YAC67 and YAC84 lines for Vegf-B gene. Brain endothelial cells (Fig. 3.17 E) revealed only minor expression changes. Vegf-A was differentially modulated in YAC67 and YAC84 and Flt4 was increased in both lines.

Presented results have shown variability of changes in endothelial cells with additional *GIRK2* gene copies. Those changes strongly differ between the lines of different origin and are not coherent with previously described FACS analysis.

Taking into account the *GIRK2* additional copies in the YAC lines the differential expression of genes that are directly related to the endothelial cell activity are significant. At this primary level of investigation it appears that profound changes are indeed the consequences of the *GIRK2* extra copies but any sound conclusion cannot be directly extracted in terms of biological behavior.

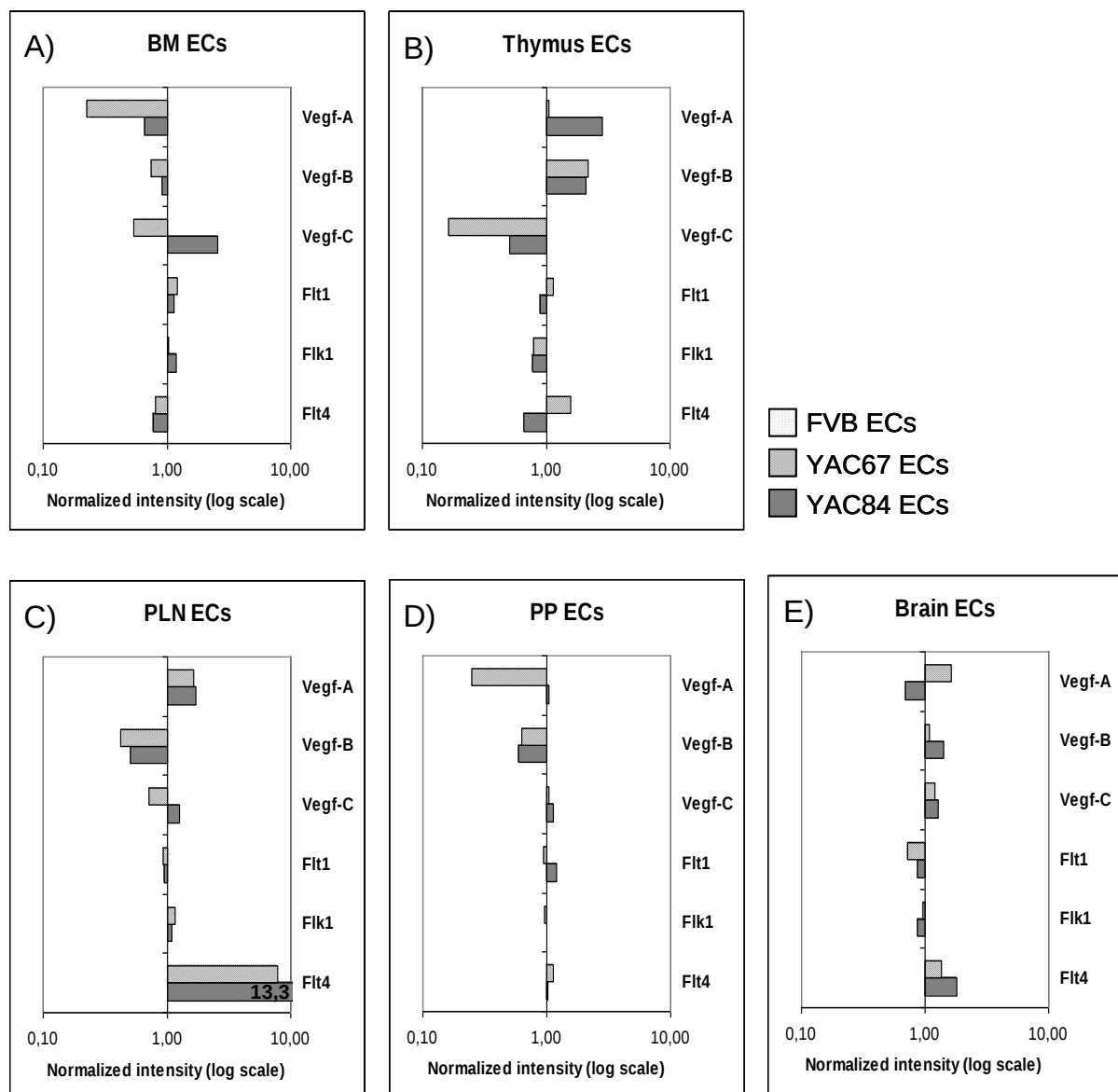


Figure 3.17. Vegf and Vegf' receptors gene expression profile of mouse endothelial cells from transgenic as compared to control mice

Vegf and Vegfr gene expression profile of bone marrow (A), thymus (B), PLN (C), PP (D) and brain (E) ECs coming from transgenic mice YAC67, YAC84 in comparison to control FVB ECs was investigated by cDNA microarray analysis. Samples were tested towards the reference sample which was the mix of RNA isolated from each cell culture. Graphs show the fold range of transgenic mice-derived ECs adhesion molecules expression level (as the optical density – horizontal axis) towards the adhesion molecules expressed by FVB ECs as means for two independent experiments. Below 1 are placed the genes with expression level lower than FVB sample. Above 1 are placed the genes with expression level higher than reference sample.

4. DISCUSSION

Among the various pathological features of the Down Syndrome, is the aberration of the immune response which largely participates to the early death of the DS persons. They present strongly altered early activation, proliferation and maturation of T and B lymphocytes.

The production and early development of lymphocytes takes place in the primary lymphoid tissues: bone marrow and the thymus. B cells mature into B lymphocytes in the bone marrow, whereas T cells enter the thymus from the blood, and expand by cell division to generate a large population of immature thymocytes, which undergo positive vs negative selections before exportation. Next, secondary lymphoid tissues: lymph nodes, Peyer's patches, skin etc. provide an environment for their further activation by antigen recognition, expansion and maturation.

The process of lymphocyte recirculation and final entry into the tissues involves multiple mutual interactions between circulating lymphocytes and endothelial cells lining lymphatic and blood vessels. The sequential interactions of leukocytes with endothelium constitute the 'adhesion cascade'. It occurs in several steps starting with rolling along the vascular endothelium, followed by tight adhesion to the endothelium and finally, transendothelial migration. Each step of the adhesion cascade engages multiple interactions of adhesion molecules with their specific ligands (Butcher 1992).

Endothelial cells from different organs express distinct cell surface phenotypes including adhesion molecules expression. Blood vessels in various tissues have specialized functions, and there might be even as many different EC types as there are organs in the body (Cleaver and Melton 2003). Therefore, endothelial pathologies resulting in serious immunological disturbances, may also have tissue specificity. In DS many changes of immunological system have been reported. Early activation, proliferation and maturation of T and B lymphocytes are strongly disturbed. Other changes including altered proportions of thymocytes and T lymphocytes subpopulations and decreased number of circulating B cells have been also mentioned. Alterations in DS T lymphocytes population may be caused by an impaired selection of thymocytes in the thymus, resulting in a perturbed proportions of mature T cells in the circulation. This would be consistent with the pattern of T lymphocyte expansion in people with thymic hypoplasia due to chromosome 22q11.2 deletion (Piliero, Sanford et al. 2004). Furthermore, DS pathology is characterised by 10-20 fold increased risk of AML. On the other hand, the frequency of solid tumors in DS is considerably lower than in the general population (Hitzler and Zipursky 2005).

A murine DS model of transgenic FVB mice with additional copies of human *GIRK2* gene was created. They were YAC67 mice carrying 2 additional copies of *GIRK2* and YAC84 mice, in which the number of additional gene copies was estimated as being 4. The background FVB mice were used as a control in all comparative experiments. These transgenic mice present symptoms similar to human DS: mental retardation, heart failures and also an inefficient immune system which is characterized, among others, with an altered distribution of T lymphocyte subpopulations. The latter is manifested as a change in the proportions among mature T lymphocytes populations, such as higher level of CD4⁺TCRαβ⁺ cells, modifications in the number of cytotoxic CD25⁺ cells, as well as changed ratio of CD4⁺CD8⁺ / CD4⁺CD8⁻ cells.

Because these differences do not appear to be connected with the process of lymphocytes maturation in the thymus (Bichler 2002), we assumed that the changes could result from an abnormal thymic exportation of matured T lymphocytes, or from disturbed homing/recirculation of lymphocytes in the periphery. Both phenomena are under the control of the endothelium of a given organ or tissue, therefore it was hypothesized that endothelial cells could play an important role in the differential distribution of lymphocytes in the DS pathology. The question aroused, whether the reason of perturbed distribution of leukocytes that is found in DS persons, is due to modifications in endothelial cells adhesion molecules pattern and/or level. A biologically valid model of endothelial microvascular cell lines, representing the main primary and secondary lymphoid organs as well as brain endothelia, derived from both model transgenic DS and their parental FVB strain mice was designed to study of DS endothelial cell phenotype and their organospecificity in the terms of recognition, adhesion and homing of lymphoid cells (Paprocka, Dus et al. 2008).

At the beginning of the adhesion process circulating cells recognize adherent cells via a protein/sugar type of recognition mechanism between adhesion molecules (CD34, other addressins and selectins) and interact with them dynamically (rolling). Such short-term connections may induce further activation of the integrins (Janeway 2005). In the preliminary experiments the expression of different molecules responsible for ECs maturation and adhesion was checked on ECs isolated from bone marrow, thymus, peripheral lymph nodes, Peyer's patches and brain. The most important changes observed concerned the expression of CD34 and CD29 molecules on endothelial cells bearing additional copies of *GIRK2* gene. In ECs from bone marrow, thymus, PLN and PP, CD34 expression levels were parallel to *GIRK2* gene copies number, increasing in the order FVB<YAC67<YAC84. On the contrary,

in brain ECs the CD34 expression in transgenic cells was lower than in the control cells, thus confirming the distinct organ specificity of endothelial cells located in brain.

The level of CD34 expression on ECs originating from DS lymphatic organs confirmed also their functional differences (Fina, Molgaard et al. 1990; Pusztaszeri, Seelentag et al. 2006). Among EC lines investigated, the highest CD34 level was found on cells isolated from primary lymphatic organs: bone marrow and thymus, where the CD34 expression in BM cells was at least twice as high as in thymic endothelial cells.

CD34 is one of the key ligands for L-selectin, the adhesion molecule present on lymphocytes, participating in their initial binding to endothelium. Consequently, the thymus ECs that express higher levels of CD34 might indeed retain differently thymus maturing lymphocytes. However, CD34 is not exclusive, but the one of several L-selectin ligands. The other known are: GlyCAM-1, MAdCAM-1, and PCLP (podocalyxin-like protein). Therefore, CD34-null mice maintain virtually normal L-selectin dependent lymphocyte homing activity. Even CD34 null and GlyCAM-1 null mice show neither deficits in hematopoiesis, nor apparent abnormalities in leukocyte trafficking (Suzuki, Sano et al. 1991; Kansas 1996). These observations have led to the suggestion that sialomucins, GlyCAM-1, CD34 and PCLP can functionally 'compensate' for each other, in cells which normally co-express these molecules (Sasseti, Tangemann et al. 1998).

Adhesion experiments, performed in static conditions, demonstrated disturbed adhesive interactions of mouse lymphocytes, and T and B lymphoma cells, with DS transgenic endothelial cell lines as compared to those from normal FVB mice. It was particularly visible in the case of transgenic endothelial cells interactions with lymphoma cells, where adhesion efficiency was significantly weaker as compared to control ECs. The adhesion efficiency towards ECs from particular organs was also different. The endothelial cells isolated from bone marrow were those to which mouse lymphocytes adhered the most efficiently.

Surprisingly, the decreased adhesion level of lymphoma cells to transgenic endothelial cells was accompanied with increased level of CD34 adhesion molecule expression in all organs. Only in the case of brain ECs, lymphoma cells adhesion efficiency was in direct proportion to CD34 level. Endothelial cells of bone marrow origin express the highest amounts of CD34 adhesion molecules. However, the level of CD34 molecule is not necessarily combined with its efficient function. The CD34 level is also influenced by inflammation. The CD34 vascular expression maintained at inflammatory sites is consistent with a suggested role of this molecule in direct leukocyte traffic (Baumhueter, Dybdal et al.

1994; Schweitzer, Drager et al. 1996; Mazo and von Andrian 1999). It was established during the treatment of the HuVEC cells with the inflammatory mediators IL-1 β , interferon- γ (IFN- γ) or TNF- α (Delia, Lampugnani et al. 1993). The mechanism by which the level of CD34 increased in transgenic ECs originating from bone marrow, thymus, PLN and PP and decreased in ECs originating from brain has not yet been established. It is known that the regulation of the expression of CD34 occurs at both the transcriptional and posttranscriptional levels (Krause, 1996). The transcriptional regulation takes place due to genomic sequence upstream of the transcription start site, containing binding sites for several potential cis-acting DNA elements (such as *myb*, *myc*, *ets*, and *mzf-1*) (Krause, Fackler et al. 1996). Apart from endothelial cells, CD34 is also expressed on stem cells, leukemic cells, and on other cells (Pusztaszeri, Seelentag et al. 2006), but only on endothelial cells does its function as an L-selectin ligand. On other locations it is not appropriately postrationally modified. If vascular CD34 would not display the appropriate carbohydrate modifications, it could not support lymphocyte trafficking to PLN. Moreover, it has been suggested that CD34 glycosylation is different in distinct organs, for example in PLN and MLN compared to non-PLN or non-MLN vessels. Apart from organ dependence, it has to be noticed that the physiological state also may modify the CD34 glycosylation pattern. This makes the CD34 molecule a very sensitive sensor of the microenvironmental conditions.

Changes in CD29 level could influence many biological processes. Being β 1 integrin subunit, it is involved in cell migration, inflammatory response, differentiation of hematopoietic stem cells, and molecular assembly of extracellular matrix proteins as well as angiogenesis regulation. The heterozygous conditional deletion of β 1 gene in the endothelium results in abnormal vascular development and patterning, while homozygous deletion in the endothelial cells causes delayed embryo cardiac development, decreased vascularization, abnormal vascular dilation, reduction of secondary and tertiary vascular patterning and vascular branching (Lei, Liu et al. 2008). Brown *et al.* hypothesized that the conformation of β 1 integrins is sensitive to small changes in membrane potential (Brown and Dransfield 2008). It was already established that integrin engagement either by cell binding to extracellular matrix and its components or by integrin-activating antibodies would activate K⁺ efflux, which can lead to cell hyperpolarization, adhesion and spreading. Inwardly rectifying potassium channels are activated by hyperpolarization. Their role lies in passing K⁺ ions into the inward direction, causing depolarization and moving membrane potential towards resting potential (Baranowska, Kozłowska et al. 2007). As a member of inward-rectifying potassium channel family, GIRK2 is expected to decrease hyperpolarization and

consequently reduce adhesion. It is supported by the fact that outward K^+ channel blockers inhibit cell adhesion. However, it was also reported that the depolarization of lymphocyte and macrophage membrane potential can also result in the activation of $\beta 1$ integrins (Brown and Dransfield 2008). The changes in CD29 expression in transgenic ECs as compared to control cells support the hypothesis that the membrane potential influences on the integrin activity.

The angiogenic abilities of transgenic endothelial cells were examined in *in vitro* test, using culture on MatrigelTM, which resembles *in vivo* conditions (Kleinman and Martin 2005). Transgenic endothelial cells were able to proliferate, but had disturbed ability to create pseudovessels in the case of cells isolated from bone marrow, thymus, and brain. Transgenic endothelial cells from primary lymphatic organs were not able to migrate and elongate forming only brushes-like structures. On the contrary, the transgenic ECs derived from secondary lymphoid organs: peripheral lymph nodes and Peyer's patches were even more elongated and created better formed pseudovessels net as compared to control FVB cells. Proper migration of endothelial cell is the integrated result of three mechanisms: chemotaxis – which is the directional migration toward a gradient of soluble chemoattractants; haptotaxis – which is the directional migration toward a gradient of immobilized ligands; and mechanotaxis – the directional migration generated by mechanical forces (Lamalice, Le Boeuf et al. 2007). Chemotaxis of endothelial cells is driven by factors such as VEGF and bFGF; haptotaxis is associated with increased endothelial cell migration activated in response to integrins binding to ECM components; mechanotaxis is driven by fluid shear stress (Lamalice, Le Boeuf et al. 2007). In the case of angiogenic assay investigated on matrix-like substratum, the disturbed migration might be the result of an improper response for chemotactic stimuli. Various cytokines can be involved. The three major stimuli of actin-based motility are VEGFs, bFGF and angiopoietins. Among the other factors that might be also responsible for disturbed migration and angiogenesis are PDGF, EGF, TGF- β , interleukins, TNF- α , platelet-activating factor, ephrins, soluble adhesion molecules, endoglin and angiogenin (Lamalice, Le Boeuf et al. 2007).

Impaired process of angiogenesis could be also the result of disturbed nitric oxide (NO) level, which role in this process was largely investigated. According to Scharbrodt *et al.* the NO level is regulated by bFGF through inward rectifier K^+ current (Scharbrodt, Kuhlmann et al. 2004). bFGF is released from macrophages and endothelial cells during hypoxia and vascular injury. It is one of the molecules regulating endothelial cells proliferation and migration. Endothelial cells treated with bFGF displayed a significant increase in the amount of inward rectifier potassium channels what consequently influenced on NO level.

This contributes also to the bFGF-mediated proliferation of endothelial cells which may provide a signaling pathway that influences angiogenesis (Scharbrodt, Kuhlmann et al. 2004).

Apart from NO level it is possible that changes in GIRK2 activity can influence the calcium ion flux. As an inwardly rectifying potassium channel GIRK2 takes part in the K^+ flux in the inward direction. As it was already mentioned inward K^+ flux changes membrane potential towards the resting potential (McCarty 1999). All changes in K^+ fluxes can have significant impact on cell biology, as K^+ channels are the major class of ion channels participating in setting the membrane potential (Nilius and Droogmans 2001). Membrane potential of endothelial cells modulates the driving force for transmembrane Ca^{2+} fluxes. A strong driving force for calcium influx is hyperpolarization of the cell membrane. Changes in inwardly rectifiers can alter Ca^{2+} fluxes. It is expected that depolarization would decrease stimulated NO production by limiting calcium influx. NO can inhibit thrombosis, atherogenesis, vasoconstriction and inflammation by a variety of mechanisms (McCarty 1999). It is also assumed that plasma membrane potential regulates endothelial production of superoxide. *In vivo* studies have shown that quenching of NO by endogenously generated superoxide is often a physiologically important mechanism that can markedly impair endothelium-dependent vasodilation and promote hypertension (Nakazono, Watanabe et al. 1991). It is already well documented that patients with Down syndrome are characterized by increased oxidative stress what is the reason of NO reduced bioavailability (Cappelli-Bigazzi, Santoro et al. 2004). Cappelli-Bigazzi *et al.* hypothesized that reduced NO amount is the reason for endothelial cell dysfunction in DS individuals. The confirmation of this hypothesis could be studies demonstrating that NO can be inactivated by vascular oxygen-derived free radicals accounted for endothelial impairment observed in patients with diabetes and coronary artery disease (Levine, Frei et al. 1996; Ting, Timimi et al. 1996).

Angiogenesis and homing are the processes which comprise many transcription factors, chemokines, and stimulating or inhibiting cytokines. To investigate the multiple factors influencing angiogenesis process as well as lymphocyte homing, complementary DNA microarray analysis was chosen. This modern technology allows for simultaneous examination of thousands genes function. Therefore, this should allow to understand the changes in biology of endothelial cells with additional *GIRK2* copies in a more detailed manner. cDNA derived from an experimental and a reference RNA samples were analyzed in parallel, after a single hybridization, on the same array, thereby giving a direct comparison of genes expression level between transgenic and control ECs as well as between ECs originating from distinct organs. Initial analysis of obtained results, presented in this thesis,

includes the verification of expression pattern of chosen genes. These genes were divided into three groups: adhesion molecules, chemokines and chemokines' receptors, and VEGF with VEGF's receptors. Among investigated adhesion molecules mRNA expression levels, 26% were at least twice smaller or twice higher than in the normal FVB control. In the case of chemokines and chemokines' receptors these percentages were calculated as 27% for YAC67 and 24% for YAC84 cells. As for VEGFs and VEGF's receptors group, in YAC67 cells the expression of 20% genes was changed as compared to 13% of genes in YAC84 cells. Above mentioned estimations point out the complexity of changes in cells possessing additional copies of only one gene, and the percentages of those changes are not higher in YAC84 cells carrying more *GIRK2* copies than YAC67 cells.

Unexpectedly, results obtained after DNA microarray data analysis do not confirm results obtained by immunochemistry. There are several assumptions which could explain this discrepancy. The first and already known is the fact that alterations in gene expression measured at the transcript level might not always accurately reflect alterations in protein levels (MacKay, Li et al. 2004). Protein formation is controlled on many levels like DNA transcription, RNA splicing, RNA stabilization, export, translation, protein folding and its export to the surface of the cell. Some of these regulation steps concerning CD34 adhesion molecule, were already discussed above. Other explanation could be the differences in protein formation, like in the case of integrins, which could exist in different affinity states. In response to certain agents, such as divalent cations or antibodies, that bind to their extracellular domain and change their conformation, integrins can function in high or low affinity state. Integrin can also respond to internal cellular signals that could have an impact on its cytoplasmic domain ("inside-out signalling") (Aplin, Howe et al. 1998). Discrepancy between the immunocytochemical cell labeling results and DNA microarray results establishing RNA level could be also caused by microarray technique itself. Despite the progress in microarray technology there are still multiple sources of variation that could compromise reproducibility, including differences in probe sets, data normalization methods, tissue sampling, and differences among the populations studied (Pusztai 2006).

Another interesting point still to elaborate is to expand these studies by examining the ability of the DS model endothelial cells to respond to hypoxia, versus normoxia. This could bring new insights in the knowledge of DS related defects that may be sensitive to the partial pressure of oxygen and the redox microenvironmental cell regulation. Disturbed process of angiogenesis by the hypoxia signalling pathway can contribute to development of pathologies, such as Alzheimer disease, amyotrophic lateral sclerosis, diabetic neuropathy,

stroke, atherosclerosis, hypertension, or diabetes. Diminished angiogenesis in the case of bone marrow, thymus and brain ECs is coherent with the fact, that the solid tumors in patient with DS are very rare. Lack of angiogenesis could be one of the factors inhibiting tumors growth.

DSCR-1 (Down syndrome candidate region-1) is one of the candidate genes which is expected to be partially responsible for the low percentages of solid tumors in DS patients. Transgenic mice with additional copy of *DSCR-1* gene hve inhibited growth of Lewis lung carcinoma and B16F10 melanoma cells with corresponding decrease in microvessel density, as compared with tumors from wild-type littermates. Endothelial cells isolated from those mice exhibited decreased sensitivity to VEGF relatively to wild-type endothelial cells, as assessed by VEGF-induced proliferation (Baek, Zaslavsky et al. 2009).

Because human chromosome 21 contains over 200 genes, it would be surprising if *DSCR-1* was the only gene on chromosome 21 implicated in tumour suppression in Down's syndrome individuals. Other genes which could be involved in the angiogenesis disturbance are *DYRK1*, *ETS2* (Baek, Zaslavsky et al. 2009) and presented in this thesis *GIRK2*.

Concluding, *GIRK2* overexpression can have complex consequences in DS pathology. When it comes to endothelial cells from different organs all of them indicate strong abnormalities. Additional copy or copies of *GIRK2* potassium channel can be the reason of changed phenotype and physiology of endothelial cells. As shown with the help of the cellular model here designed, this effect depends upon the endothelial cell origin. With our animal model and isolated endothelial cell lines, the causes of Down syndrome immunological abnormalities could be, at least partially, uncovered.

CONCLUSIONS

Down syndrome pathology is accompanied with many disorders, one of them is the impaired immunological system. The changes involve strongly disturbed early activation, proliferation and maturation of T and B lymphocytes as well as altered proportions of thymocytes and T lymphocytes subpopulations, and decreased number of circulating B cells. All these alterations result from, at least partially, by abnormal thymic exportation of matured T lymphocytes and/or by disturbed recirculation of the lymphocytes in the periphery. Because both phenomena are under the control of endothelium it may be hypothesized that endothelial cells play a role in the DS pathology. Recent findings describing endothelial disturbances in transgenic mice with additional copy of Down syndrome critical region-1 support this hypothesis.

A model of two strains of transgenic mice: YAC67 and YAC84, carrying 2 or 4 copies, respectively, of *GIRK2* gene which is one of the genes present in the Down syndrome critical region, was created. These mice present symptoms similar to human DS. In the presented thesis a panel of endothelial microvascular cell lines, isolated from different organs of transgenic and wild type FVB mice, was used to study the DS endothelial cell phenotype and their organospecificity.

As it was demonstrated, the main differences between transgenic and control endothelial cells concerned the expression of some adhesion molecules, leukocytes adhesion efficiency and the angiogenic abilities of ECs. These differences could be summarized as follows:

1. The expression of CD34 and CD29 molecules was changed. In ECs from bone marrow, thymus, PLN and PP, CD34 expression levels were parallel to *GIRK2* gene copies number, increasing in the order FVB<YAC67<YAC84. On the contrary, in brain ECs the CD34 expression in transgenic cells was lower than in the control cells. As concerns CD29 molecule, the transgenic EC lines from primary lymphoid organs and non-directly-related organ, as from the brain, expressed less CD29 as compared to the secondary lymphoid organs. Peripheral lymph node derived ECs and, particularly, Peyer's patches derived cells displayed an increased expression of CD29 in both YAC67 and YAC84 versus FVB control cells. On the contrary, CD29 expression on thymus, bone marrow and brain ECs derived from transgenic mice is slightly lower than on ECs from control mice.

2. Adhesion experiments demonstrated disturbed adhesive interactions of mouse lymphocytes and T and B lymphoma cells, with transgenic endothelial cell lines as compared to those from normal FVB mice. In the case of transgenic endothelial cells interactions with lymphoma cells, the adhesion efficiency was significantly weaker as compared to control EC cells. The adhesion efficiency towards ECs from particular organs was also different. The endothelial cells isolated from bone marrow were those to which mouse lymphocytes adhered the most efficiently.
3. Transgenic endothelial cells had disturbed ability to create pseudovessels in the case of cells isolated from bone marrow, thymus, and brain. Transgenic endothelial cells from primary lymphatic organs were not able to migrate and elongate forming only brushes-like structures. On the contrary, the transgenic ECs derived from secondary lymphoid organs: peripheral lymph nodes and Peyer's patches were even more elongated and created better formed pseudovessels net as compared to control FVB cells.
4. The mRNA expression level, measured by the microarray technique, was also altered in transgenic versus control ECs. Among investigated adhesion molecules, 26% had at least twice smaller or twice higher mRNA expression levels than in the normal FVB control. In the case of chemokines and chemokines' receptors these percentages were calculated as 27% for YAC67 and 24% for YAC84 cells. As for VEGFs and VEGFs' receptors group, in YAC67 cells the expression of 20% genes was changed as compared to 13% of genes in YAC84 cells.

The above demonstrated results show that overexpression of only one gene connected with DS pathology (*GIRK2* gene) may cause complex disturbances in endothelial cells phenotype and functions. These changes depend also on the endothelial cells origin what confirms their organospecificity.

Down syndrome is characterized by an increased risk of acute leukemia but the frequency of solid tumors is considerably lower as compared to the general population. It may be hypothesized that endothelial cell biology affected by DS hamper tumor vascularization and, in consequence, tumor progression. Determination of additional factors influencing tumor vascularization in DS may create new approach to tumor treatment.

SUMMARY

GIRK2 is one of the 200 genes located on chromosome 21, which trisomy is the cause of a Down syndrome. Down syndrome manifests itself, among other features, by immunological disturbances, such as impaired activation, proliferation and maturation of T and B lymphocytes as well as altered proportions of thymocytes and T lymphocytes subpopulations and decreased number of circulating B cells. These changes could result from an abnormal thymic exportation of matured T lymphocytes, and/or from disturbed homing/recirculation of lymphocytes in the periphery. Both these processes are controlled by the endothelial cells, located at the internal surface of blood vessels.

Endothelial cells interact with circulating cells and initiate the process of adhesion and immunological response. Apart from adhesive interactions, ECs participate in new vessels development. To verify if the *GIRK2* gene may influence the function of endothelial cells, an *in vitro* cellular model was established. Those cells were isolated from transgenic mice with additional copies of the gene, which are believed to be a model of DS. Isolated endothelial cell lines biology was investigated in the aspect of adhesion molecules as well as processes of adhesion and angiogenesis.

As it was shown in the present thesis, ECs from transgenic mice have altered level of CD29 and CD34 adhesion molecules. The level of CD34 molecule was increased in cells isolated from bone marrow, thymus, peripheral lymph nodes, Peyer's patches, but decreased in the cells isolated from brain. Moreover, the levels of adhesion molecules differed between the ECs from particular organs in both transgenic as well as control ECs.

The adhesive interactions toward ECs isolated from transgenic mice have been also affected. T and B lymphoma cells showed significantly decreased adhesion efficiency toward transgenic ECs, similarly T and B lymphocytes isolated from FVB mouse have shown changed adhesion efficiency towards transgenic ECs. These changes varied greatly in the aspect of the *GIRK2* copies as well as the ECs origin.

The most pronounced differences were found in angiogenic properties of endothelial cell lines tested. Transgenic ECs isolated from bone marrow, thymus and brain displayed disturbed angiogenesis process, whereas transgenic ECs isolated from peripheral lymph nodes and Peyer's patches create more developed pseudovessels net as compared to control cells. The changes in angiogenesis are parallel with the number of additional *GIRK2* copies.

Using cDNA microarray technique the selected gene expression pattern of ECs from transgenic mice was investigated. Among adhesion molecules, chemokines and chemokines'

receptors, VEGFs and VEGFs' receptors tested more than one fourth of the concerned mRNA was significantly over or under expressed compared to the FVB controls. However, those changes were not coherent with the results obtained from flow cytometry analysis.

Although *GIRK2* is only one out of many genes located on chromosome 21, presented results give clear evidence that this one gene can influence the function of endothelial cells in DS persons. This influence implies for the important role of adhesion and angiogenesis in DS pathology.

Changed phenotype and reactivity of endothelial cells confirm the hypothesis that the endothelium dysfunction can be the reason of altered distribution of T lymphocyte populations in mice possessing additional copies of *GIRK2* gene. Future investigations concerning the mechanism of these changes may give additional knowledge of the general mechanism of Down syndrome pathology and enable for searching of pharmacological solutions eliminating effects of additional *GIRK2* copy as well as other genes presence and activities.

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Magdalena TOMCZYŃSKA

Biologie de l'endothélium vasculaire isolé de souris transgéniques YAC67 et YAC84- modèles murins du syndrome de Down

Résumé: *GIRK2* est situé sur le chromosome 21, dont la trisomie cause le syndrome de Down (DS). Les proportions des sous-populations de lymphocytes T sont altérées, le nombre de lymphocytes B circulants est diminué. Notre hypothèse est un défaut de contrôle de la domiciliation/recirculation des leucocytes par les cellules endothéliales (CE).

Les CE formant la paroi des vaisseaux, assurent la néovascularisation, interagissent avec les cellules circulantes, initient l'adhésion donc, la réponse immune.

Pour élucider l'influence de *GIRK2* sur la fonction des CE, un modèle cellulaire *in vitro* a été mis au point. Des lignées de CE furent établies à partir de: moelle osseuse, thymus, ganglions lymphatiques périphériques, plaques de Peyer et cerveau de souris transgéniques dotées de copies additionnelles du gène et de souris contrôles. La biologie de l'endothélium fut abordée quant aux molécules d'adhésion, et processus d'adhésion et d'angiogenèse.

Les CE issues des souris transgéniques expriment différents niveaux de CD29, CD34, leurs propriétés d'adhésion des lymphocytes ainsi que d'angiogenèse sont dramatiquement affectées.

Le profil d'expression des gènes des CE de souris transgéniques montrent que parmi les molécules d'adhésion, chimiokines et récepteurs, VEGFs et récepteurs, plus d'un quart des ARNm est considérablement modifié par rapport aux contrôles.

Nos résultats montrent clairement que le gène *GIRK2* influence la fonction endothéliale des patients atteints de DS.

Mots clés : Syndrome de Down, cellules endothéliales, *GIRK2*

Biology of vascular endothelium isolated from transgenic mice YAC67 and YAC84 -mouse models for Down syndrome

Summary : *GIRK2* is located on chromosome 21, which trisomy is the cause of Down syndrome (DS). In DS, among other features, proportions of T lymphocytes subpopulations are altered and number of circulating B cells are decreased. We hypothesized that it is due to the disturbed control of homing/recirculation of lymphocytes by endothelial cells (ECs).

ECs constitute the vessel wall, achieve the neovascularisation, interact with circulating cells, initiate the adhesion process thus, immunological response. To assess the *GIRK2* gene influence on the function of ECs, an *in vitro* cellular model was established. ECs lines were established from bone marrow, thymus, peripheral lymph nodes, Peyer's patches and brain from transgenic mice with additional copies of the gene and from normal control mice. Endothelium biology was investigated in the aspect of adhesion molecules as well as processes of adhesion and angiogenesis.

ECs from transgenic mice have altered levels of CD29, CD34, their adhesive properties towards lymphoid cells are affected and their angiogenic properties are drastically different.

cDNA microarray display for the gene expression pattern of ECs from transgenic mice showed that among adhesion molecules, chemokines, chemokine receptors, VEGFs and VEGFs receptors, more than one fourth of the mRNA was significantly modified compared to controls. Presented results give clear evidence that *GIRK2* gene can influence the function of endothelial cells in DS patients.

Keywords : Down syndrome, endothelial cells, *GIRK2*

Centre de Biophysique Moléculaire

Institute of Immunology and Experimental Therapy

