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A systems biology approach to Down syndrome: Identification of Notch/Wnt dysregulation in a model of stem cells aging

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ABSTRACT

Stem cells are central to the development and maintenance of many tissues. This is due to their capacity for extensive proliferation and differentiation into effector cells. More recently it has been shown that the proliferative and differentiative ability of stem cells decreases with age, suggesting that this may play a role in tissue aging. Down syndrome (DS), is associated with many of the signs of premature tissue aging including T-cell deficiency, increased incidence of early Alzheimer-type, Myelodysplastic-type disease and leukaemia. Previously we have shown that both hematopoietic (HSC) and neural stem cells (NSC) in patients affected by DS showed signs of accelerated aging. In this study we tested the hypothesis that changes in gene expression in HSC and NSC of patients affected by DS reflect changes occurring in stem cells with age. The profiles of genes expressed in HSC and NSC from DS patients highlight pathways associated with cellular aging including a downregulation of DNA repair genes and increases in proapoptotic genes, s-phase cell cycle genes, inflammation and angiogenesis genes. Interestingly, Notch signaling was identified as a potential hub, which when deregulated may drive stem cell aging. These data suggests that DS is a valuable model to study early events in stem cell aging.

1. Introduction

Stem cells are central to the development, maintenance and repair of all tissues. This is due to their capacity for extensive proliferation and differentiation into multiple effector cells. The fact that aging is accompanied by a diminished capacity to adequately maintain tissue homeostasis has suggested that a decline in stem cell function may be central to the process of tissue aging.

Indeed stem cells from several tissues have been shown to functionally decline with advancing age. In hematopoietic stem cells (HSC) this is manifested with a decreased competitive repopulating ability, a skewing of lineage potential from lymphopoiesis to myelopoiesis [1]. This is thought to contribute to the loss of immune function [2], increased incidence of leukaemia [3] and onset of anaemia [4] occurring with age. Similarly neural stem cells (NSC) have been shown to be reduced in numbers and proliferative potential in the subventricular zone and in dentate gyrus of the hippocampus with age [5–7]. Moreover diminished neurogenesis has been observed in the olfactory bulb of old mice [5], where NSC migrate and contribute to neurogenesis. Neurogenesis is thought to be important for sensory and cognitive functions such as memory and learning [8], and therefore NSC aging has been linked to the decline of those activities in older people.

Understanding genes and their interactions leading to stem cell aging is vital to open up opportunities for drug discovery and strategies to identify compounds capable of extending tissue survival and repair. Preventive targeting of relatively young stem cells, which show predisposition to an accelerated aging is more likely to be successful rather than targeting of aged stem cells which have undergone profound and complex changes and unlikely to be reverted by any intervention. Given that aging is considered a continuous process starting early in development and occurring at different pace in each individual the identification of when and whose stem cells require intervention is difficult but key to the design of any therapy.

We have previously identified Down syndrome (DS) as a model to study early events occurring in stem cells with age. DS is associated with many of the signs of premature tissue aging including early abnormalities typical of Alzheimer disease (AD), T-cell deficiency, increased incidence of Myelodysplastic-type disease and leukaemia

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[9–13]; and can be detected from an early phase in development thanks to the presence of trisomy 21. We have shown that stem cells in DS show signs of premature aging. Mean telomere restriction fragment length (mTRF) of peripheral blood lymphocytes declines more rapidly in individuals with DS than in normal individuals reflecting an accelerated HSC telomere shortening [14]. The accelerated telomere shortening is already present in fetal life and is associated with stem cell deficiency as shown by a reduction in cells possessing the phenotype of HSC (detected as CD34+ cells) in fetal blood and bone marrow (BM) of DS children and in the number of long term culture initiating cells in their BM [14]. Moreover NSC derived from the cortical tissue of DS fetuses at 17–19 weeks of gestation show severely reduced replicative capacity and early loss of neuronal differentiation capacity after 10 weeks in culture [15].

In this study we have used HSC and NSC from patients affected by DS at very early stages of development, to identify early changes in gene expression occurring in HSC and NSC with age. Using a combination of genomic analysis and mathematical modelling, we have identified a dysregulation of the Notch/Wnt pathway and showed that changes in DS stem cells reflected molecular events occurring in stem cells of older people. These data are consistent with the hypothesis that DS is an invaluable model to determine the molecular markers predisposing to stem cell aging and is suitable to unveil new molecular targets for intervention.

2. Materials and methods

2.1. Sample collection and cell separation

Following a protocol approved by the local research ethics committees and in agreement with the Helsinki declaration of 1975, bone marrow was obtained from the iliac crest of DS children (1–5 years) with no clinical hematological abnormalities undergoing cardiac surgery and from age matched hematologically healthy children during similar significant surgery following parental consent. Bone marrow was also collected from individuals 60–80 years old clear from any hematological malignancy. Long term hematopoietic stem cells were isolated by labeling mononuclear cells with CD45 APC, CD34 PE and CD38 FITC (BD biosciences) according to manufacturer's instructions. CD45+CD34+CD38− (200–1000 cells) were sorted on FACS Aria sorter (Becton Dickenson) following the addition of propidium iodide (10 μg/ml, Fluka) to exclude dead cells. The gating strategy used is represented in Fig. 1. Purity was assessed at the end of the sort whenever cell numbers allowed and was found to be >95%.

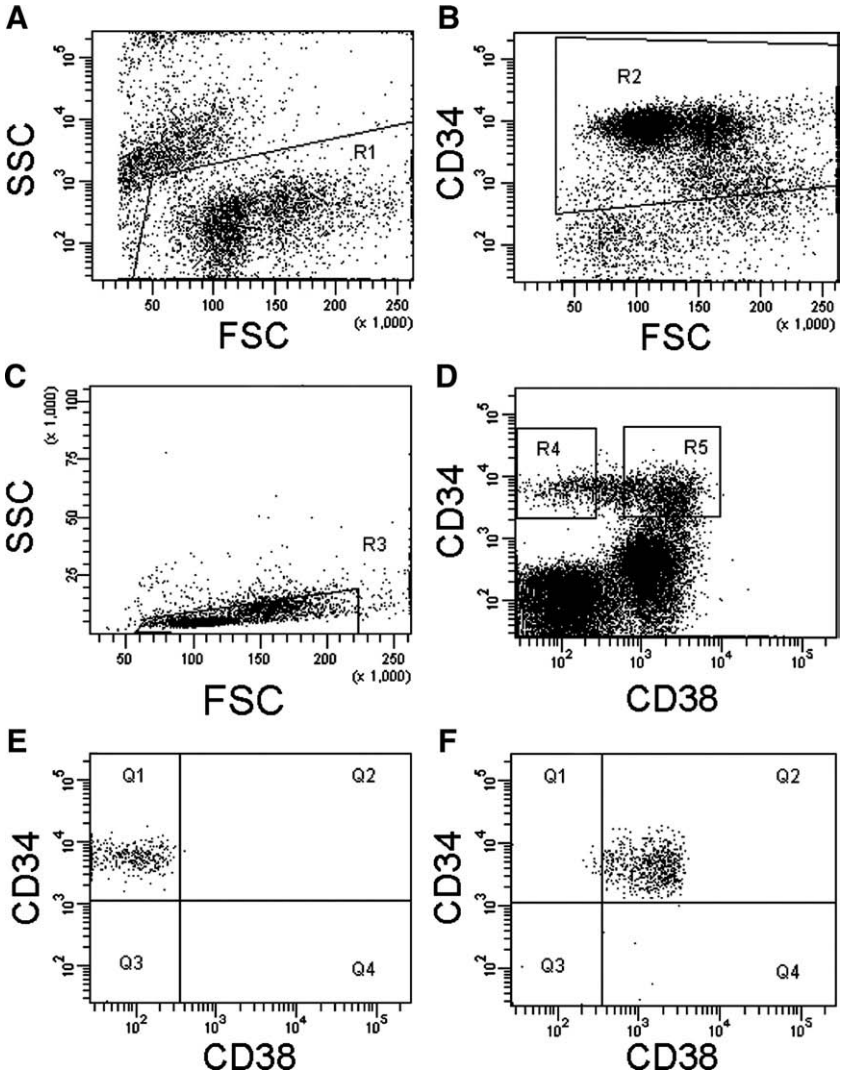


Fig. 1. Representative example of the gating strategy used to obtain PI−/CD45+/CD34+/CD38− HSC. (A) A region for live bone marrow cells (PI negative) was drawn (R1); (B) Cells contained in the R1 region were plotted and CD45+ cells (leucocyte cells) were further selected in the R2 region; (C) The cells contained in both the R1 and R2 regions were plotted and a R3 region was drawn around the lymphocyte population; (D) The cells common to all three regions were plotted for CD34 and CD38 expression and the CD34+ and CD38− population was identified (R4) and sorted; (E) CD34+ CD38− population after sort; (F) CD34+ CD38+ population after sort. The purity was measured and found to be >95%.

For the isolation of NSC human fetal tissue was obtained from the Birth Defects Laboratory at the University of Washington, Seattle and the Tissue Bank for Developmental Disorders at the University of Maryland. Neurospheres were generated from two trisomy 21 cortex samples and two gestationally age matched controls (12 and 18 weeks gestation). The method of collection conformed to the guidelines recommended by National Institutes of Health for the collection of such tissues and set out by the University of Washington, the University of Maryland and the University of Wisconsin, Madison. Institutional Review Board approval was obtained for all of these studies. Cortical precursors isolated from fetal brain were induced to proliferate as free-floating neurospheres [16]. Briefly, freshly isolated tissue was mechanically chopped and seeded into flasks. The cells were initially expanded in DMEM/Ham's F12 media with penicillin, streptomycin, amphotericin B (PSA, 1%) and supplemented with B27 (2%; Life Technologies) and mitogens: EGF (20 ng/ml; Sigma), and FGF-2 (20 ng/ml; R & D Systems) with heparin (5 μg/ml). Media were replenished every 3–4 days. Neurospheres were passaged every 14 days by a chopping method that does not require trypsin or mechanical dissociation, and cell–cell contact was continuously maintained [16]. After 2 weeks, neurospheres were grown in DMEM/Ham's F12 media with penicillin, streptomycin, amphotericin B (PSA, 1%) and supplemented with N2 (1%; Life Technologies) and 20 ng/ml EGF.

2.2. PolyA RT-PCR

A total of 200–1000 HSC or NSC were directly lysed in cell lysis buffer and PolyA RT-PCR was carried out. Global amplification of *cDNA* corresponding to all expressed genes (polyA PCR) was carried out as previously reported [17]. Control samples in absence of reverse transcriptase were also generated.

2.3. Microarray analysis

cDNA samples obtained from 200 cells were labeled using EpiLabel™ (Epistem, UK) developed by Brady et al. [17,18] and hybridisided to the Affymetrix HU133 plus 2.0 chip (Affymetrix). Microarray analysis was carried out at the Paterson Institute for Cancer Research microarray facility. Background levels, intensities of spike hybridisation controls were within the acceptable range for all samples. The percentage of present calls was $25.2 \pm 3.0\%$ for HSC and $37.6 \pm 1.5\%$ for NSC. Quality controls can be viewed at http://bioinformatics.picr.man.ac.uk/vice/StartRegistration.vice.

2.4. Data analysis

Normalization was carried out by using the Simpleaffy implementation [18]. The dendogram was generated based on Pearson correlation scores and hierarchical clustering using Bioconductor (Stats package, http://www.bioconductor.org/).

Identification of age related genes was carried out based on the hypothesis that DS samples show accelerated aging when compared to normal samples and that aging is a continuous process with changes, which start early in development and increase with time. Since the NSC cells are from a much earlier stage of development, a simple model to explain the differences between HSC samples and NSC samples was built as

$$\Delta X_{\mathrm{HSC-NSC}} = \Delta + \varepsilon$$

where X is the gene expression level, the first term on the right hand side is the difference due to the different developmental stages, and the second term includes various sources of noise (including the fact that the samples come from different tissues). Under the hypothesis that DS is a model for accelerated aging, we use the difference in ΔX between normal and DS samples to detect candidate markers for aging. We empirically estimated the variance of $\alpha = \Delta X_{\mathrm{DS}} - \Delta X_{\mathrm{N}}$ and selected probes exhibiting a signal to noise ratio greater than 2 equivalent to a p-value of 0.05.

Transcriptional regulation and pathway analysis was performed using Metacore software (GeneGo Inc.). Analysis of transcriptional networks was performed using the transcriptional regulation network building algorithm. This algorithm generates subnetworks centred on transcription factors, which regulate the most number of genes on the input list. All positive and negative interactions from manually curated published data were included in the analysis.

Gene interaction subnetworks of the Notch signaling pathway containing high numbers of differentially expressed probes were analysed using the MMG tool [19, 20]. Briefly, MMG searches the network for coherently expressed subnetworks by employing a statistical technique, the Markov Random Field. This essentially encodes the intuitive idea that a gene interacting with many differentially expressed genes is more likely to be differentially expressed. Genes with a probability of being differentially expressed greater than 80% are assigned to the differentially expressed class, and subnetworks are then obtained by exhaustive percolation search.

Table 1
Primers' sequence used for RT-qPCR

Gene	Forward	Reverse
EGR1	GTGATGCGCCTTGCTGATG	CTCCCTGCCCCCCTTAAG
IL8	TCCTAGTTTGATACTCCCAGTC	ATTGACTGTGGAGTTTTGGC
KLF4	AGCCTAAATGATGGTGCTTGGT	CCTTGTCAAAGTATGCAGCAGTTT
JAG1	TCACTGTTTAGATTTGCCATAGAGTACA	ATAAGCCTTTTTGATCTTGAACTTCGT
Beta cat	TGGACAGTTTACCAGTTGCCTTT	TAACCGCATTTTCTCTTGAAGCA
c-Fos	TGTACTGTAGTTTTTCTTCAACATCAATG	TTCATGGAAAACTGTTAATGTCAGAAC
Smad2	GGATGCAGACCCTGTTCTTGTT	TAATATCTCCATCACAGTGCACCAA
DAB2	TGCAAATGAGGGAAGAACATTATTC	GGAGCAAAACTGACTGAAAAAAGAA
P21	CAGCGACCTTCCTCATCCA	CCTTGTTCCGCTGCTAATCAA

2.5. Real time quantitative PCR

PCR primer pairs were designed for mRNA sequence within 500 bp of the 3′ end of each gene (Table 1) using Primer Express Software (Perkin Elmer/Applied Biosystems) and used in PCR reactions carried out in 10 μl containing qPCR Mastermix for Sybr Green I (Eurogentec), 0.1 μM reverse and forward primers and 2 μl of 1:100 or 1:1000 dilution of PolyA cDNA. PCR was performed using the following thermal cycle: 2 min 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 60 s at 60 °C. Samples were analysed using an ABI Prism 7700 sequence detection system (PE Applied Biosystems) as recommended by the manufacturer. A dilution series of human genomic standards for calibration of real-time PCR was generated using human genomic DNA (Promega). Only primers with an efficiency coefficient of −3.3 to −3.8 were considered. The expression level of the L32 gene was used to normalize for differences in input of polyA amplified cDNA. Each sample was run in triplicate and a RT-PCR negative control was also tested to exclude any contaminating DNA amplification. The expression ratio was calculated using the standard curve method and calculating amounts for each sample normalized to L32 amounts. Differences were considered significant with $P<0.05$ following non parametric one way ANOVA Kruskal Wallis test.

3. Results

3.1. Selection of differentially expressed genes

The ability of HSCs to permanently reconstitute myeloablated recipients in all blood cell lineages is the most rigorous criteria for evaluating HSC activity. In human, the long-term reconstituting HSC reside within the CD34+/CD38- fraction of cells in the BM [21]. Total

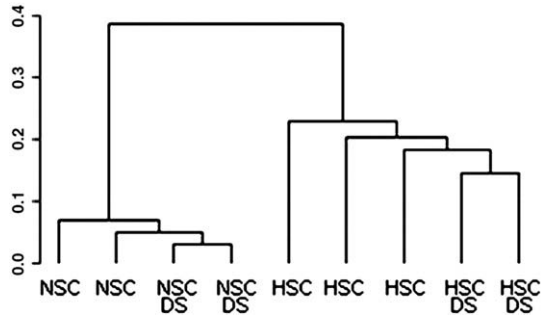


Fig. 2. Cluster dendrogram of gene expression of NSC and HSC from patients affected by DS and age matched controls. Data clustered by Pearson correlation and hierarchical clustering.

RNA from 200 PI−/CD45+/CD34+/CD38− cells from patients affected by DS but with no apparent hematological abnormalities (HSC-DS $n=2$) and healthy age matched controls, (HSC, $n=3$) was amplified. Neurosphere cultures derived from the cortex of primary human DS fetal tissue (NSC-DS, $n=2$) at 12 and 18 weeks gestation respectively, before neuroanatomical defects are observed, and age matched healthy controls (NSC, $n=2$) were established. Total RNA from 200 cells was used for amplification before any change in their growth kinetic and differentiation capacity had ensued and gene expression profiling carried out by Affymetrix gene chip.

Hierarchical clustering of the samples using the expression profiles of all genes showed clustering between the two different cell types (Fig. 2). Moreover samples obtained from healthy donors had similar expression patterns and clustered together but were separate from samples obtained from donors affected by DS, suggesting differences between healthy controls and patients affected by DS in both stem cell types, although those differences were not very pronounced.

Gene expression data from all samples were combined according to the model described in Materials and methods to detect age-related differential expression. Under an assumption of Gaussian noise on the log expression levels, we identified possible up and downregulated genes by considering those whose expression levels in the two conditions differed by more than three standard deviations (estimated from the data under normal assumptions). With this analysis we have found 430 genes which were differentially expressed, 192 genes were downregulated and 238 upregulated. Of these, 149 upregulated genes (Supplementary data Table 1) and 103 downregulated genes (Supplementary data Table 2) had known information on function, regulation and interaction with other genes. We used this selected shortlist for further analysis.

Analysis of the shortlist of differential genes in terms of biological function using the manually curated pathway analysis software Metacore revealed 30 statistically overrepresented networks (Fig. 3). Most of the pathways listed have been previously associated with aging. One mechanism believed to be central to the aging of cells is DNA damage (Fig. 2, process n3) [22]. The downregulation of genes involved in DNA repair (FANCM, Sirtuins and DNA polymerase eta) suggests that stem cells from patients affected by DS are more susceptible to accumulation of DNA damage. Depending on the extent of the damage, the DNA lesions arising in the stem cells have the potential to drive cells into senescence or apoptosis. Indeed the upregulation of proapoptotic genes Grim19 and Ft1 or iTRAF, which blocks the antiapoptotic function of TRAF proteins implies an imbalance in the apoptotic process in favour of cell death (Fig. 3,

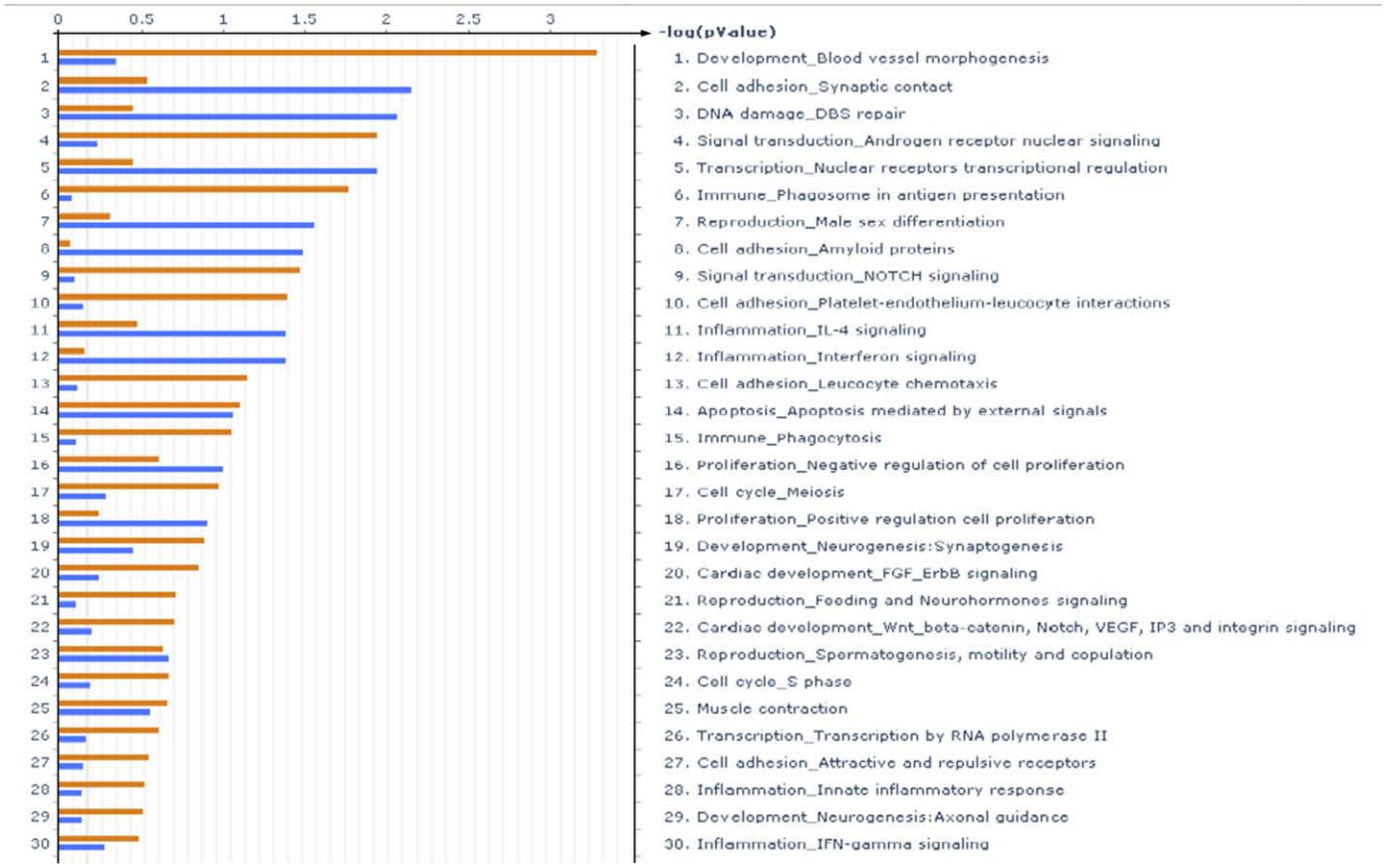


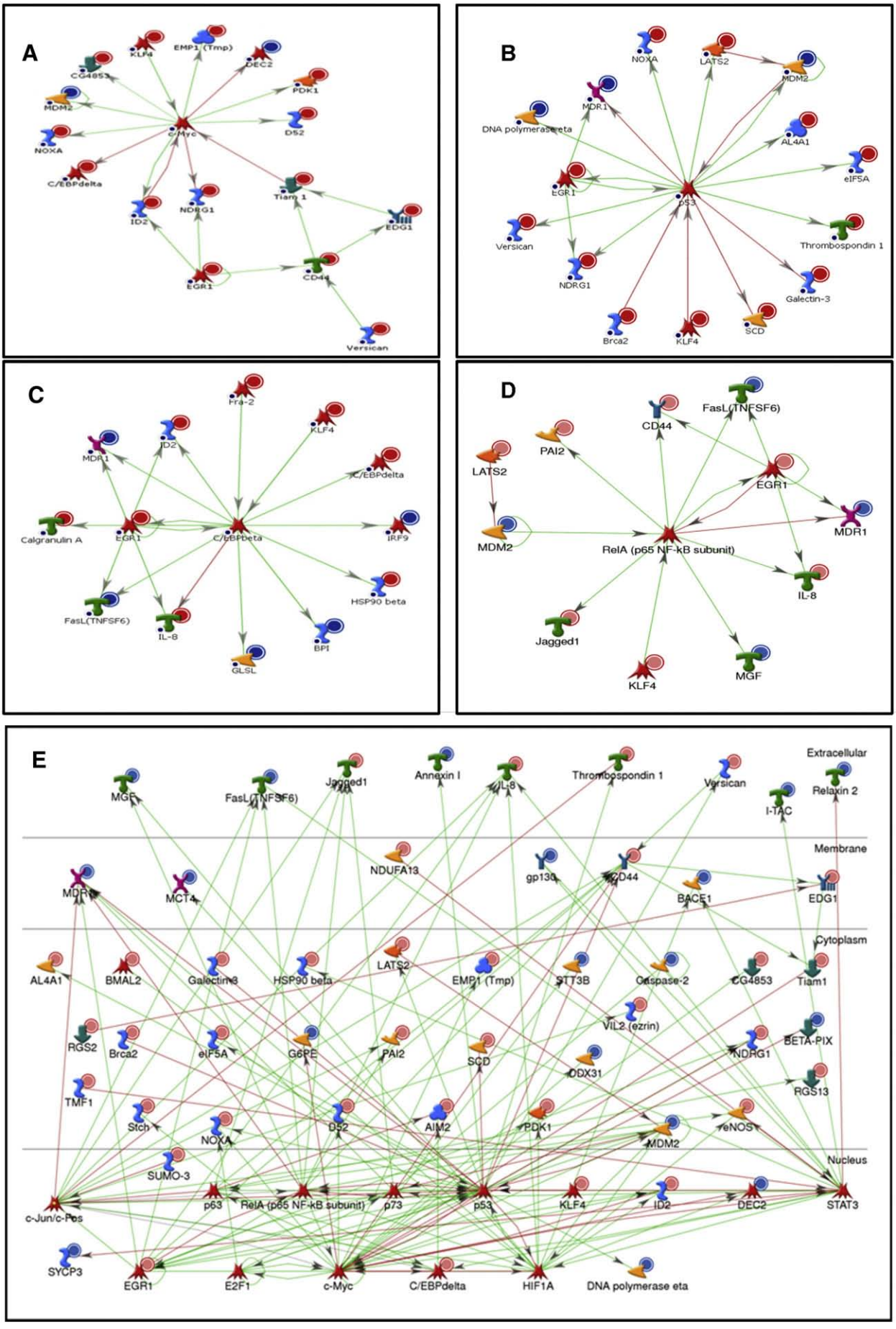
Fig. 3. Biological process networks overrepresented in the shortlist of genes upregulated (orange) and downregulated (blue) in Down syndrome compared to age matched controls. Enrichment analysis was performed in Metacore pathway-mapping software. Log of *P*-values represent the probability of a given number of upregulated and downregulated genes being associated with each network by chance.

process n. 14). The enrichment in genes which promote S phase of the cell cycle (Fig. 3, process n. 24) and genes of the Notch pathway (Fig. 3, process n. 9), known to promote self-renewal [23], suggests the possibility of stem cells undergoing compensative proliferation. The presence of numerous pathways involved in inflammation is highly intriguing and in line with very recent data where two biomarkers of human aging, chitinase and CRAMP, were identified in the mTERC−/− mouse model of telomere dysfunction and DNA damage and then validated further in the serum of patients with chronic diseases and telomere shortening [24]. These proteins are involved during activation of innate immunity and inflammatory disease and suggest that the immune system may be activated by DNA damage and contribute to the clearance of apoptotic or senescent cells. Of interest and surprising at first is the strong enrichment in genes involved in angiogenesis such as IL8, VEGF, amphiregulin, angiogenin, Jagged1 or HIF1 [25–27]. All of these genes have been described as major players in changing the tumor microenvironment to enhance the survival and proliferation of tumor cells [25–28]. It is tantalizing to speculate that stem cells are capable of changing their own niche through secretion of factors which increase neovascularisation to suit their survival and proliferative needs. Overall these data suggest a model where stem cells in DS showed decreased ability to repair damage, with consequent accumulation of DNA damage leading to enhanced apoptosis and establishment of an inflammatory and highly vascularised environment. The remaining stem cells undergo increased proliferation in a cytokine rich environment to compensate losses.

Table 2
Significant transcriptional networks associated with the highest number of genes differentially expressed in stem cells of patients affected by DS compared to healthy age matched controls

Transcriptional network	Processes	G score	*P* value	Molecules in the network
c-Myc	Regulation cell cycle	58.94	1.43^{-37}	KLF4, EMP1, DEC2, PDK1, D52, Tiam1, EDG1, CD44, Versican, NDRG1, EGR1, ID2, C/EBPdelta, NOXA, MDM2, CG4853
SP-1	Senescence, DNA damage, differentiation of HSC	56.95	3.33^{-35}	EGR1, FasL, SLC39A8, LIPA. eNOS, KLF4, MGF. BMP2, Alpha-1D adrenergic receptor, PAI2, GLSL, MDM2, LATS2, BACE1, C/EBPdelta, CYP1B1
P53	Tumor suppression, senescence	56.95	3.33^{-35}	Noxa, LTS2, MDM2, AL4A1, eIFSA, Thrombospondin, galactin-3, SCD, KLF4, Brca2, NDRG1, Versican, EGR1, DNA polymerase beta, MDR1
ESR1 (nuclear)	Regulation of mitosis and survival	54.90	7.71^{-33}	Rab-31, Thrombospondin 1, Amphiregulin, RNF32, gp130, HERC6, NOXA, OCTN2, CYP1B1, Fra-2, C/EBPdelta, MDM2, LATS2, MDR1. BMP2
Androgen receptor	Regulator of Notch, Wnt, p53	54.90	7.71^{-33}	IL-8, EGR1, NDRG1, Caspase-2, LATS2, MDM2, PGAR, NOXA, TMF1, Brca2, Versican, VIL2, Sry, CDK11
C/EBP beta	Modulation of cell cycle, T and B cell development, senescence	52.76	1.77^{-30}	KLF4, cEBPbeta, IRF9, HSP90 beta, BP1, GLSL, IL8, FasL, EGR1, Calgranulin, MDR1, ID2, Fra-2
HIF1A	Blood vessel development, directly interact with Notch, regulation of telomerase	50.54	4.02^{-28}	EGR1, MDR1, ID2, BACE1, MGF, PDK1, DEC2, MCT4, eNOS, MDM2, LATS2, IL-8, BMAL2
STAT3	Early response to cytokine and inflammation, promotes HSC self-renewal under stimulated conditions, regulator of hTERT expression	50.54	4.02^{-28}	eNOS, NDUFA13, I-TAC, Relaxin 2, C/EBPdelta, HSP90beta, FasL, IL-8, gp130, TMF1, BACE1, SYCP3
RelA (p65 NK-kB subunit)	Positive regulation of Notch signalling	48.22	9.04^{-26}	EGR1, MDR1, Il8, PAI2, KLF-4, jagged1, MDM2, MGF, CD44, FasL
STAT1	Retention of balanced hematopoiesis	45.78	2.01^{-23}	KLF4, IRF9, BACE1, MDM2, gp130, Caspase-2, SP100, HSP90, C/EBPdelta, FasL
GCR-alpha	Regulator of transcription	45.78	2.01^{-23}	HSP90 beta, Relaxin 1, NURR1, Relaxin 2, TR-beta1, MGF, IL-8, FasL, EGR1, NOR1
c-Jun	Apoptosis, cell growth and differentiation	43.21	4.41^{-21}	eNOS, jagged1, thrombospondin, PAI2, FASL, EGR1, IL8, MDR1, C/EBPdelta
P63	Stem cell proliferation, differentiation, senescence, regulator of Notch	37.57	2.02^{-16}	LATS2, MDM2, Jagged1, AIM2, RGS13, C/EBPbeta, CD44
FOXO3A	Regular cellular resistance to stress, trigger DNA repair, regulate HSC self-renewal	34.42	4.22^{-14}	eNOS, FasL, TANK, KLF4, MDM2, LATS2, MDR1
Runx2	Regulator of bone commitment	34.42	4.22^{-14}	RGS2, Galectin-3, C/EBPdelta, ID2, EGR1, MDR1
C/EBPalpha	Myeloid development, growth arrest	30.98	8.56^{-12}	EGR1, GPD1, BP1, C/EBPdelta, Annexin 1
P73	Positive regulation of Notch signalling, regulation of telomerase	30.98	8.56^{-12}	EGR1, MDR1, jagged1, MDM2, LATS2
ETS1	Stem cell development, cell senescence and death, tumorigenesis	30.98	8.56^{-12}	IL-8, SUMO-3, eNOS, MEIS1, SP100, MDR1
Progesterone receptor	Bone remodelling	27.14	1.68^{-9}	TR-beta2, Relaxin 2, Relaxin 1
NF-AT1 (NFATC2)	Control T cell activation	27.14	1.68^{-9}	FasL, MDM2, LATS2, BACE1
Elk-1	Induction of immediate early gene response (i.e. Fos, EGR1), senescence	27.14	1.68^{-9}	EGR1, ID2, SUMO-3, eNOS
c-Fos	Positive regulation of Notch signaling, cell growth, differentiation, transformation	27.14	1.68^{-9}	IL-8, EGR1, VIL2, Jagged1
PU.1	Myeloid cell differentiation, B and T cell development, HSC maintenance	27.14	1.68^{-9}	EGR1, ID2, KLF4, BPI
SF1	Adrenal gland development	22.75	3.16^{-7}	CYP1B1, Sry, EGR1
c-Jun/c-Fos	Tumorigenesis, modulator of apoptosis, cell growth, differentiation	22.75	3.16^{-7}	IL-8, SUMO-3, CD44
HMGI/y	Regulator of transcription	22.75	3.16^{-7}	EGR1, MGF, CD44
NF-kB1 (p50)	Inflammation, apoptosis	22.75	3.16^{-7}	FasL, HBXAP, IL-8
E2F1	Cell proliferation, apoptosis, cell differentiation	22.75	3.16^{-7}	NOXA, MDM2, LATS2
NUR77	Apoptosis, transformation	22.75	3.16^{-7}	FLAD1, EGR1, Fra-2
STAT5A	Self-renewal of HSC, maintenance of normal and leukaemic stem cells	22.75	3.16^{-7}	TR-beta1, Relaxin 2, Brca2

P value for each network indicates the probability of assembly from a random set of genes.


A
KLF4
EMP1 (Tmp)
DEC2
CG4853
PDK1
MDM2
DS2
c-Myc
NOXA
C/EBPdelta
Tiam 1
ID2
NDRG1
EDG1
EGR1
CD44
Versican
B
NOXA
LATS2
MDM2
MDR1
DNA polymerase eta
AL4A1
eIF5A
EGR1
DS3
Versican
Thrombospondin 1
NDRG1
Galectin-3
Brca2
KLF4
SCD
C
Fra-2
KLF4
MDR1
ID2
C/EBPdelta
Calgranulin A
EGR1
C/EBPbeta
IRF9
HSP90 beta
FasL(TNFSF6)
IL-8
BPI
GLSL
D
CD44
FasL(TNFSF6)
PAI2
LATS2
EGR1
MDR1
MDM2
RelA (p65 NF-kB subunit)
IL-8
Jagged1
KLF4
MGF
E
Extracellular
Membrane
Cytoplasm
Nucleus
MGF
FasL(TNFSF6)
Jagged1
Annexin I
IL-8
Thrombospondin 1
Versican
I-TAC
Relaxin 2
NDUFA13
gp130
CD44
BACE1
EDG1
MDR1
MCT4
AL4A1
BMAL2
Galectin-3
HSP90 beta
LATS2
EMP1 (Tmp)
STT3B
Caspase-2
CG4853
Tiam1
VIL2 (ezrin)
RGS2
Brca2
eIF5A
G6PE
PAI2
SCD
DDX31
NDRG1
BETA-PIX
RGS13
TMF1
Stch
NOXA
DS2
AIM2
PDK1
MDM2
eNOS1
SUMO-3
c-Jun/c-Pos
p63
RelA (p65 NF-kB subunit)
p73
p53
KLF4
ID2
DEC2
STAT3
SYCP3
EGR1
E2F1
c-Myc
C/EBPdelta
HIF1A
DNA polymerase eta

3.2. Transcriptional network analysis

Transcription factors are the major players in governing stem cell self-renewal and differentiation and as such may constitute the master regulators of the aging-related expression profile. However due to the low level of their expression or to the modification occurring at the post-translational level during their activation, they are often lost by microarray analysis. To overcome this problem we used the transcriptional regulation network mapping algorithm within Metacore to search for the transcription factors which regulate the highest number of genes present in the differentially expressed gene shortlist. This analysis highlighted 30 significant transcriptional networks (Table 2), which identifies important key regulators of stem cell aging in patients affected by DS and is in agreement with our analysis of biological process involvement. Transcription factors important in aging and senescence such as c-myc (Fig. 4a), p53 (Fig. 4b) and c/EBPbeta (Fig. 4c) regulated the highest number of genes on the differential list and as such were among the most significant networks [29–31]. NF-kB (RelA) (Fig. 4d), NF-AT1 and HIF-1 alpha are transcriptional networks involved in inflammation and/or angiogenesis [27, 32–34]. Moreover NF-kB is also activated by increased intracellular ROS [35] suggesting that stem cells may be subjected to increased damage. Indeed the dysregulation of FOXO3A is in support of this. FOXO3A has recently been shown to be important in the preservation of long-term HSC reserve and function with age by protecting them from ROS damage [36].

Of the 30 significant networks 10 were related to Notch signaling (p53, c-Myc, HIF1A, p63, p73, RelA, c-Jun, c-Fos, STAT3, E2F1) an important regulator of stem cell numbers in vitro and in vivo and known to promote self-renewal [23, 37]. As transcription factors related to Notch were the most represented we then searched using the direct interactions network building algorithm within Metacore for known positive and negative direct interactions among the genes involved in all 10 transcriptional networks and identified the novel network represented in Fig. 4e. This network is enriched for genes present on the differentially expressed gene shortlist and shows a great deal of connectivity between the various Notch-related transcription factor networks. As such it represents an interesting model of the signaling pathways underlying the aging profile generated from the differential gene list and a biological framework on which to further investigate stem cell aging and self-renewal.

3.3. Notch subnetwork analysis

From our previous pathway and transcriptional network analysis Notch signaling has emerged as an important player. To further identify the altered subpathway involved in the accelerated aging of stem cells in DS with age we integrated data on direct interactions among genes of the Notch pathway, provided by Metacore, with the expression data using the MMG tool as described above. The key idea underlying this is that integrating network structure should yield a more informative way of detecting the differential expression state of genes. The input for the model was the values of $\alpha = \Delta X_{DS} - \Delta X_N$ for the 161 genes involved in the pathway. The output of the model is a set of posterior probabilities of regulation class for the genes: 47 genes had posterior probability greater than 0.8 of being up-regulated in DS, and 28 had posterior probability greater than 0.8 of being down-regulated in DS. Percolation analysis of the network revealed four coherently regulated subnetworks (two up-regulated and two down-regulated); the genes involved in these networks, as well as their Affymetrix probe IDs and their fold changes (values of α), are listed in Table 3 and represented in Fig. 5.

Table 3
Subnetworks identified using the MGG tool

Gene	Probe ID	$\alpha = \Delta X_{DS} - \Delta X_N$
Network 1		
MAPK14	211087_x_at	−2.52
JUN	203752_s_at	−1.77
FOS	218880_at	−6.83
FRZB	203697_at	−2.44
PTEN	211711_s_at	−2.23
ARNT	218222_x_at	−5.84
EPAS1	200878_at	−3.17
HIF1A/HIF	200989_at	−1.72
CTNNB1	201533_at	−2.20
DVL	57532_at	−1.70
FZD	204451_at	−2.76
WNT	208570_at	−3.61
SFRP4	204051_s_at	−2.04
DAB2	201280_s_at	−4.12
SMAD4	202527_s_at	−2.25
SMAD2	201280_s_at	−2.36
VEGFA	210512_s_at	−3.13
EP300	202221_s_at	−1.82
JAG1	231183_s_at	−5.56
RBPJ	211974_x_at	−2.35
HEY1	218839_at	−2.80
Network 2		
ERBB2	222473_s_at	−2.31
ERBB3	226213_at	−4.63
GRB2	215075_s_at	−2.62
SHC1	214853_s_at	−1.27
SOS	1557354_at	−2.70
Network 3		
HRAS	219984_s_at	2.15
RAF1	1557675_at	2.90
BRAF	206044_s_at	1.90
MAP3K1	214786_at	3.50
MAP2K3	215498_s_at	2.31
MAP2K7	216206_x_at	4.14
MAPK8	213014_at	3.69
p53	223920_s_at	2.92
MYC	216188_at	3.16
Network 4		
ERBB4	206794_at	2.57
NRG1	208241_at	3.72
EGF	206254_at	3.77
TGFA	211258_s_at	2.19
EGFR	211607_x_at	3.77

Overall the subnetwork analysis supports the model suggested by the analysis of the biological processes. Increased proliferation is suggested by most networks. Activation of HIF1A and VEGF is in line with the transcriptional network analysis and reinforces the concept of increased neovascularisation of the microenvironment as an autocrine mechanism to improve survival and proliferation. Similarly the increase in the expression of ErbB-2 has been shown to be associated with recruitment of progenitor cells during the cell-cycle [38]. Most important is the upregulation of key molecules belonging to Notch, WNT and TGFbeta pathways, all shown to be involved in self-renewal of HSC and NSC [39]. Indeed the cooperation between WNT and Notch has recently been reported as important for HSC maintenance [40] and molecules such as beta catenin and Smad4 have been shown to have a role in HSC maintenance and self-renewal

Fig. 4. Transcriptional regulation of the shortlist of genes up and downregulated in Down syndrome compared to age matched controls. Transcriptional networks showing genes regulated by A — c-myc, B — p53, C — C/EBP alpha and D — RelA transcription factors. E — Direct interactions network showing connectivity between genes in the 10 transcriptional networks related to Notch: RelA, p53, c-fos, p73, p63, c-jun, c-myc, HIF1A, STAT3 and E2F1. The genes represented with a red solid circle were found to be upregulated in HSC from DS patients, while those with a blue circle were downregulated. Green lines indicate positive interactions, while red lines indicate negative interactions. Network objects are separated out according to their sub-cellular location.

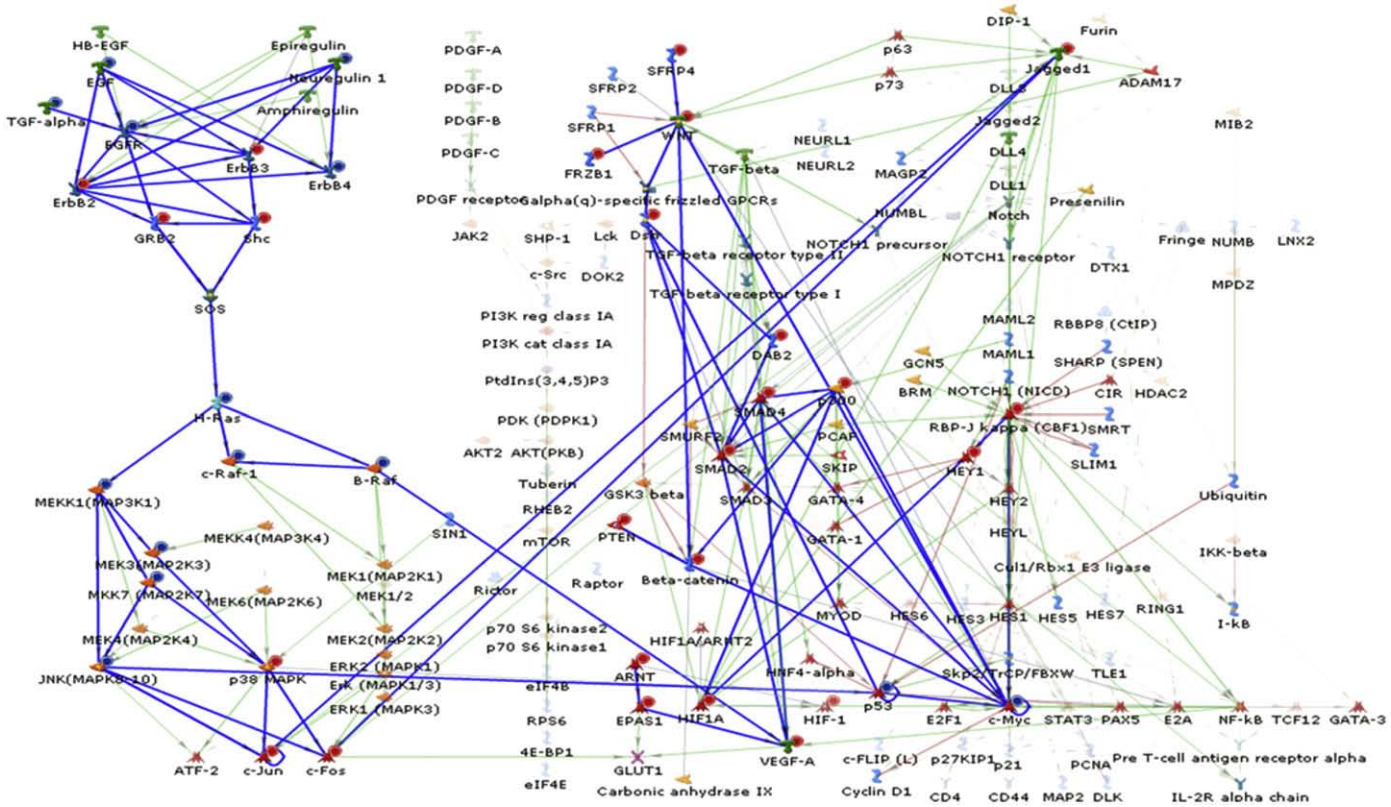


Fig. 5. Representation of significant subnetworks in the notch pathway. "Signal transduction_Notch signaling" GeneGo process static network drawn in Metacore and overlayed with genes from the 4 significant subnetworks described in Table 3. Connectivity between genes involved in these subnetworks is represented by blue lines. The genes represented with a red solid circle were found to be upregulated in HSC-DS (networks 1 and 2 in Table 3), while those with a blue circle were downregulated (networks 3 and 4 in Table 3).

in murine models. Overexpression of beta catenin has been shown to prevent HSC differentiation and promote proliferation leading to higher levels of chimerism in transplantation [41]. Smad4 deficient HSC displayed a significantly reduced repopulation capacity in primary and secondary recipients, suggesting that increased levels promotes HSC self-renewal [42]. A decrease in c-myc and p53 expression seen in network 3 (Table 3) is in favour of preservation of the stem cell pool either by blocking differentiation [43] or increasing proliferation [44]. However, increased DNA damage accompanied by increased proliferation potentially poses a danger for malignant transformation especially in a cytokine rich environment. Indeed it has been shown that mice with a reduced expression of p53 showed an increase in the HSC pool but also an increase in the incidence of tumorigenesis [44]. It is possible that a decrease of genes such as Pten [45] or increase of p38MAPK [46] is put in place as censor mechanisms to block the rise of potentially malignant clones by inducing apoptosis or senescence.

3.4. Validation of DS as model of stem cell aging

To test whether changes in expression seen in stem cells of patients affected by DS reflected changes in stem cells of older people we performed quantitative RT-PCR (qRT-PCR) on key genes present on either or both of the networks identified by transcriptional analysis and MGG analysis (represented in Figs. 4 and 5). We compared expression in PI−/CD45+/CD34+/CD38− HSC of hematologically healthy 1–5 years old donors ($n=7$) with expression in HSC of age matched patients affected by DS ($n=4$) and 60–80 year old donors ($n=9$). According to the developmental model of aging, changes in expression were expected to be greater when comparing HSC of younger and older donors. Only two genes, PAI2 and LATS, were upregulated in DS but not in HSC of older people. All the remaining genes tested are shown in Fig. 6 and follow the same trend in HSC of patients affected by DS and HSC of older donors. Of interest is that the changes in expression in HSC of patients affected by DS were mostly at intermediate levels between the expression levels of HSC in younger and older healthy donors.

The disadvantage of this method is that it is only able to account for positive interactions and therefore pathways are expected not to be exhaustive. Indeed p21 an important gene of the pathway, which senses telomere attrition and block cell proliferation in response to telomere dysfunction was found upregulated in the expression array but did not emerge by any of the network analyses. The upregulated expression was confirmed by realtime qPCR on independent DS HSC and HSC of older people, confirming that further work is required for the complete identification of the pathways involved. These data suggests that DS detects early events occurring in stem cells with age and that age is an incremental process that starts during fetal development and continues throughout life. Notch and Wnt are important in the process of human stem cell aging but some data are missing and are for further study.

4. Discussion

This study shows how a systems biology approach based on modelling of the process of stem cell aging and strengthened by information on direct protein interactions in networks and pathways can overcome some of the limitations imposed by previous gene expression analysis and lead to the identification of a stem cell aging signature.

To date gene expression studies of human stem cells had been carried out to identify a stem cell self-renewal signature but failed in their intention [47–49]. The limitation of those studies is in the analysis based on simple bioinformatics and comparison of gene lists

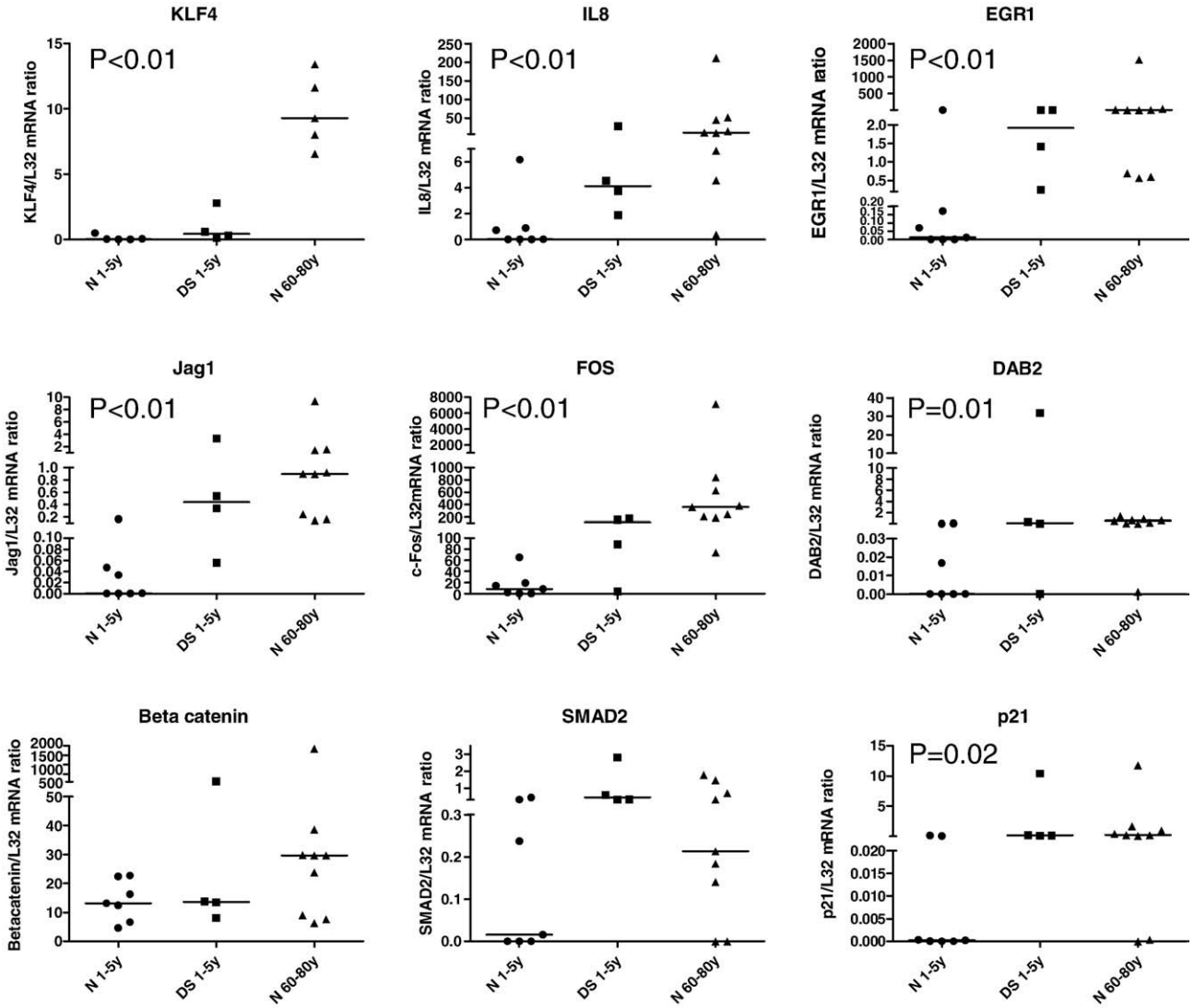


Fig. 6. Genes which are differentially expressed in DS follow the same pattern in HSC of older people. Realtime qPCR for the expression of KLF4, IL8, EGR1, JAG1, FOS, DAB2, Beta catenin, SMAD2, p21 in PI−/CD45+/CD34+/CD38− cells from hematologically healthy younger donors (1–5 years old), patients affected by DS (1–5 years old) and hematologically healthy older donors (60–80 years old). *P* values are indicated when statistical significance was reached by ANOVA.

without taking into consideration the underlying biology. In contrast, to select the differentially expressed genes our study considers a model of aging whereby all stem cells in a pool have similar properties and gradually and co-ordinately change over time during development and aging. The model predicts that DS patients changes in NSC (fetal stem cells) are smaller than those seen in HSC (adult stem cells) due to their earlier stage of development. Moreover, to enrich for biologically significant processes we have considered only the differentially expressed genes of known function and used experimental information on connectivity between proteins, their subunits and DNA sequences. In this way we were able to identify key transcription factors, independently of post-translational modification, and important subpathways involved in the accelerated aging of stem cells of patients affected by DS.

The data obtained show strength in a variety of ways. There is great consistency of the results among the different analyses carried out. Transcriptional networks and subnetworks identify molecules which overlap and are central to processes related to aging. Most importantly key genes identified by our analysis were also identified by two other studies in a different progeroid syndrome, the Hutchinson Guilford Progeroid Syndrome, and in a different stem cell type, the mesenchymal stem cell [50,51], highlighting how these pathways are well conserved among stem cell types and across accelerated aging disorders and may well be the core molecules of an aging stem cell signature. Although most of the genes we have identified are not included in the differentially expressed genes identified in HSC of older mice by bioinformatic analysis [1], mining of the same data for individual genes such as c-Fos, KLF4 and IL8 showed upregulation of these genes in mice with age, suggesting that those networks may well be conserved between human and mice and a similar signature could be found if the same mathematical model was applied.

We have focused the validation of DS as a model of stem cell aging to the analysis of HSC due to their easier availability from older donors where, according to the model, changes were expected to be the greatest. We have shown that 9/11 genes tested and expected to be overexpressed in HSC of patients affected by DS were also up-regulated by HSC of older people. These data suggest that overall changes in HSC of patients affected by DS reflect changes in HSC of older people. As predicted by the mathematical model those changes were even greater in HSC of older donors than in HSC of patients affected by DS because they occurred in a later phase of development, in line with a model of continuous development of the aging process and with DS as a model of early changes in aging.

This study supports the attempt to preserve the stem cell pool by upregulation of genes active in enhancing proliferation or inhibiting differentiation to compensate for losses due to increased damage, apoptosis and senescence. However, in agreement with the view that advancing age is accompanied by an increased incidence of cancer and that stem cells represent the ideal target for the accumulation of premalignant damage, it also supports the notion of a tenuous balance between tumor suppression and stem cell aging. There are several elements which suggest that the compensative proliferative stress may put the stem cell at higher risk of neoplastic transformation. The decrease in p53 levels may signal a stem cell which is predisposed to malignant transformation. p53+/− mice showed an increase in HSC numbers but also an increase in the incidence of cancer [44]. Decrease Pten levels have been associated with the generation of leukaemia initiating cells [45]. Active NK-kB has been found to correlate with proinflammatory cytokine and proangiogenic factors to form an environment favourable to survival and proliferation but also to malignant transformation [33,52]. On the other hand factors such as C/EBPbeta have been shown to decrease proliferation and induce senescence in response to oncogenic stimuli to restrict the expansion of incipient neoplastic cells, leading to activation of multiple interleukins such as IL8 [31]. Indeed IL8 expression has been seen to increase 1000 fold in response to oncogenic stimuli [31]. The presence of tumor suppressor mechanisms triggered by telomere dysfunction, such as those mediated by the increase of p21, seems to counter-balance the oncogenic potential of the aged stem cell and its environment. However any failure of those mechanisms may find favourable conditions for cancer development.

In summary this is the first study to identify key genes and processes occurring in human stem cells with age and validates Down syndrome as an excellent model to detect early events in aging of stem cells. It is the first time that molecules such as p38 MAPK, FOXO3A and p21, which have been shown to have a role in stem cells with age using artificial murine knock out models, do play a role in the regulation of HSC of older individuals in physiological conditions. Identification of upregulation of molecules such as p21 provides hope that aging may be delayed without concomitant increase in cancer incidence by the careful manipulation of the p21 pathway in stem cells as shown in murine models [53].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbadis.2009.01.015.

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