Bio-Medical Materials and Engineering 24 (2014) S53–S61
DOI 10.3233/BME-140974
IOS Press

Gene screening of Wharton's jelly derived stem cells

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Abstract. Stem cells are the most powerful candidate for the treatment of various diseases. Suitable stem cell source should be harvested with minimal invasive procedure, found in great quantity, and transplanted with no risk of immune response and tumor formation. Fetal derived stem cells have been introduced as an excellent alternative to adult and embryonic stem cells use, but unfortunately, their degree of "stemness" and molecular characterization is still unclear. Several studies have been performed deciphering whether fetal stem cells meet the needs of regenerative medicine. We believe that a transcriptomic screening of Wharton's jelly stem cells will bring insights on cell population features.

Keywords: PCR array, human Wharton's jelly stem cells, regenerative medicine

1. Introduction

Stem cells (SCs) have been considered among the most promising cell populations for regenerative medicine. SCs can be defined as pluripotent or multipotent cells which have the capability of self-renewal and the potential to differentiate into several lineages. SCs can be classified into embryonic, fetal or adult stem cells that are further divided mainly into hematopoietic and mesenchymal stem cells (MSCs) [1]. Notably, SCs are characterized by secretion of a wide spectrum of molecules with immunomodulatory, anti-inflammatory, proangiogenic, proliferative or chemoattractive capacities. All these features confer very promising potential to these cells for clinical purposes [2–4].

Despite the promising characteristics of pluripotent stem cells (embryonic and induced SCs), there are some obstacles to overcome before their clinical application such as the assurance of guided differentiation, control of the immune response and cell misbehaviour following cell grafting. MSCs can be isolated from a wide variety of adult tissues including bone marrow, adipose tissue, dental pulp... with the disadvantages of an invasive harvesting, painful procedure and their mitigated therapeutic efficiency

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linked most probably to the age and health of the donor [3]. Fetal tissues, sustaining natal development, and discarded after delivery, represent a safe and non-invasive way for SCs harvesting, in addition to the fact that they are non-controversial sources. These tissues include amnion/amniotic fluid, umbilical cord blood, placenta and full-term umbilical cord. Fetal stem cells are considered more proliferative and even more therapeutically active than adult SCs [4]. Stem cells from Wharton jelly (WJ, a gelatinous substance that insulates umbilical blood vessels and most studied fetal SCs) express all the typical MSCs markers that have been established by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy [5]. Despite the expression of specific MSCs markers, some pluripotent markers such as *Oct4*, *Nanog*, *Sox2* and *Lin28* have been reported [6]. However, inconsistencies regarding protocols of WJ derived stem cells harvesting and characterization still exist and to date, this is one of the main obstacles to extend their clinical applications. In this study, we report a random screening of gene transcripts for WJ stem cells at the fourth passage.

2. Materials and methods

2.1. Wharton's jelly derived cells isolation and culture

Human umbilical cords were obtained after full-term births with informed consent using the guidelines approved by the University Hospital Center of Reims. After rinsing in Dulbecco's Phosphate Buffered Saline (DPBS, *Gibco*, France), umbilical cord was cut into segments of 1–2 cm of length with sterile scissors. Each piece was then cut open lengthwise and blood vessels were then removed allowing the peel-off Wharton's jelly. Cells were harvested using a two-step enzymatic digestion of isolated Wharton jelly according with Fong et al. [7]. Briefly, the first step consists of an overnight digestion with type-II collagenase (2 mg/ml, *Fisher*, France) followed by 30 min of trypsin digestion (0.5 p/v, *Gibco*, France). Wharton's jelly derived cells were cultured in α-MEM (*Lonza*, Belgium) supplemented with 10% decomplemented fetal bovine serum (FBS), penicillin/streptomycin/amphotericin (100 IU/ml), Glutamax® (2 mM) (all from *Gibco*, France) and maintained in a humidified atmosphere with 5% CO_2 at 37°C. Submitted to three passages, cells were morphologically examined by scanning electron microscopy, phenotypically characterized by flow cytometry or dry-stored at −80°C until RNA extraction.

2.2. Real-time PCR analysis

Total RNA was extracted from −80°C dry-stored WJ-SCs using MasterPure™ RNA Purification Kit (*Epicentre® Biotechnologies*, France) in accordance with the manufacturer technical data sheets. RNA purity was assessed by measuring the absorbance ratio at 260 nm/280 nm (*Nanodrop®, Thermo Scientific*, France). All measured samples were comprised between 1.9 and 2. RNA was reverse transcribed using RT2 first strand kit (*Qiagen*, France) following manufacturer instructions. After reverse transcription, the cDNA product was amplified by quantitative real-time PCR (qRT-PCR). PCR was performed using RT2 Profiler PCR Array – Human mesenchymal stem cells array (*Qiagen*, France), using a 96-well format. All reactions were performed on an ABI 7000 thermalcycler (*Applied Biosystems*, UK). Data analysis was performed with the 7000 SDS software (*Applied Biosystems*, UK). Results were calculated in accordance with the following formula: 2^{35-Ct}. Detection limit of qRT-PCR technique was considered to be the Ct value of 35. Based on this threshold, expression levels under 128 copies from the detection limit were considered as low expression, expressions levels between 128 and 16,384 copies from the detection limit were considered as moderate expression and finally, data beyond 16,384 copies from the detection limit were considered as high expression.

3. Results and discussion

Caplan et al. described MSCs as patient-specific drugstores for injured tissue because of a huge secretion panel of growth factors and other chemokines able to induce cell proliferation and angiogenesis, to modulate inflammatory and immunological reactions and to rescue apoptotic cells [8]. In this study, using enzymatic digestion of Wharton jelly tissue, cells were extracted then amplified until fourth passage. Scanning electron microscopy examinations and flow cytometer analysis showed that all morphological features and immunophenotypic surface markers match with the basic criteria for multipotent phenotype established by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy [9] (Fig. 1 and Table 1). In the present work, using the Human Mesenchymal Stem Cell RT2 Profiler PCR array, we assessed the expression of 78 genes (Table 2). The data showing more than 16,384 number of copy above threshold for limit of detection were considered as highly expressed genes, between 128 to 16,384 number of copy above threshold for limit of detection as moderately expressed genes, and under 128 number of copy above threshold for limit of detection as weakly expressed genes. Distribution of studied genes represented in Figs 2 and 3 showed, at the transcriptional level, a high degree of consistency between cell populations isolated from different umbilical cords. Indeed, each

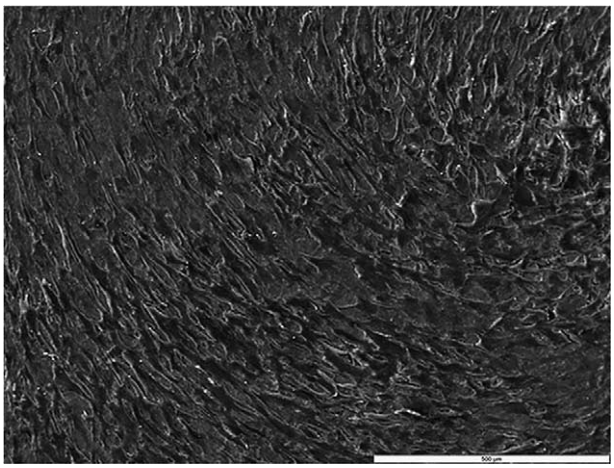

Fig. 1. Scanning electron micrograph of WJ derived stem cells cultured population. Note the fibroblastic (*spindle shaped*) morphology of the cells. Magnification $\times 100$, white scale bar $=$ 50 μm. LaB_6 electron microscope (JEOL JSM-5400 LV, JEOL).

Table 1
Flow cytometry analysis results of WJ derived stem cells

Marker	Expression
CD90	+
CD105	+
CD73	+
CD44	+
CD146	+
VEGFR2	−
CD34	−
CD31	−

category (low, medium or high expression) exhibited the same genes in most of cell-derived umbilical cord (six studied umbilical cords).

3.1. Stem cell characterization through the transcriptomic profile

First, we analyzed the gene expression of stem cells markers (Fig. 3(A)). We found a high mRNA expression of ITGB1, THY1, CD44 and while mRNA expression of ALCAM, NT5E, ENG and ANPEP seemed to have a moderate expression level. These genes are described as mesenchymal stem cells markers [9]. At a lower level, we found the expression of POU5F1, SOX2 and TERT corresponding to pluripotent stem cells markers. Unlike pluripotent stem cells, Wharton's jelly derived stem cells have not been related to teratoma induction *in vivo* [6]. Low mRNA expression of pluripotency markers could explain the absence of tumor formation after injection of Wharton's jelly derived stem cells. Negligible inter-individual variability for most of the stem cells markers indicates that the gene expression profile within the extracted cells was invariable. This confirms also that our isolation protocol is robust for stem cells isolation and a relatively homogenous stem cells population could be isolated with negligible differences in their transcriptomic profiles.

3.2. Self-renewal and undifferentiated state

Stem cell self-renewal and maintenance refers to biological pathways that preserve their undifferentiated state. Several studies demonstrate that LIF, FGF and Wnts among others mediators are implicated in self-renewal. FGF2, LIF and FGF10, WNT3A mRNA were expressed by WJ derived stem cells at different rates with respectively high, moderate and low level (Fig. 3(B)). The stem cell culture and *in vitro* expansion influence their selfrenewal properties. Although the mRNA targets are present, detection of the corresponding protein expression must be investigated.

3.3. Master genes of ectoderm-, mesoderm- and endoderm-derived cells

It is well known that WJ-SCs express genes found in early development and genes associated with the three principal germinal layers, namely, ectoderm, mesoderm and endoderm. Stem cells expressing such genes are generally able to differentiate into germinal layer-derived cells. We therefore looked for the mRNA expression of mesodermal (COL1A1, RUNX2, SOX9, BGLAP, BMP, PPARG, VEGFA, VWF), endodermal (HNF1) and ectodermal (NES, GDF7, NGFR, VIM, INS) derived cell lineages (Fig. 3(C)). Among mesodermal markers, a high mRNA expression of COL1A1 and a moderate mRNA expression of VEGFA, RUNX2, SOX9, BMP4, 6 and BGLAP was detected while a low expression of BMP2, 7, VWF and PPARG mRNA was found. *In vitro* cell plating density has a direct influence on stem cell lineage commitment [10]. At low densities, cells have been engaged into osteogenic differentiation, conversely, high cell densities favors adipogenic lineage. Our related results could support the idea that our culture conditions favor skeletal differentiation of undifferentiated cells. Concerning endodermal and ectodermal except for VIM mRNA, no higher expression was found. Finally, low expression of hepatic lineages mRNAs INS and HNF1, a moderate expression of NES and a low expression of NGFR and GDF7 (neuronal), confirm the absence of high expression of lineage specific markers. These findings suggest that our WJ-SC populations are maintained in an undifferentiated state with however a skeletal lineage predisposition.

Table 2
Gene table of the studied transcripts in the RT2 Profiler PCR Array with the Unigene and Genbank accession numbers

Description	Symbol	Unigene	Genbank
ATP-binding cassette, sub-family B	ABCB1	Hs.489033	NM_000927
Activated leukocyte cell adhesion molecule	ALCAM	Hs.591293	NM_001627
Alanyl (membrane) aminopeptidase	ANPEP	Hs.1239	NM_001150
Annexin A5	ANXA5	Hs.480653	NM_001154
Brain derived neurotrophic factor	BDNF	Hs.502182	NM_001709
Bone gamma-carboxyglutamate (gla) protein	BGLAP	Hs.654541	NM_199173
Caspase 3, apoptosis cysteine peptidase	CASP3	Hs.141125	NM_004346
CD44 molecule (Indian blood group)	CD44	Hs.141125	NM_000610
Collagen, type I, alpha 1	COL1A1	Hs.172928	NM_000088
Colony stimulating factor 2 (granulocyte-macrophage)	CSF2	Hs.1349	NM_000758
Colony stimulating factor 3 (granulocyte)	CSF3	Hs.2233	MM_000759
Catenin	CTNNB1	Hs.476018	NM_001904
Endoglin	ENG	Hs.76753	NM_000118
Epidermal growth factor	EGF	Hs.419815	NM_001963
V-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2	ERBB2	Hs.446352	NM_004448
Fibroblast growth factor 10	FGF10	Hs.664499	NM_004465
Fibroblast growth factor 2 (basic)	FGF2	Hs.284244	NM_002006
Fucosyltransferase 1 (galactoside 2-alpha-L-fucosyltransferase, H blood group)	FUT1	Hs.69747	NM_000148
Fucosyltransferase 4 (alpha (1,3) fucosyltransferase, myeloid-specific)	FUT4	Hs.390420	NM_002033
Frizzled family receptor 9	FZD9	Hs.647029	NM_003508
Growth differentiation factor 15	GDF15	Hs.616962	HM_004864
Growth differentiation factor 5	GDF5	Hs.1573	NM_000557
Growth differentiation factor 6	GDF6	Hs.492277	NM_001001557
Growth differentiation factor 7	GDF7	Hs.447688	NM_182828
General transcription factor IIIA	GTF3A	Hs.445977	NM_002097
Histone acetyltansfrase 1	HAT1	Hs.632532	NM_003642
Histone deacetylase 1	HDAC1	Hs.88556	NM_004964
Hepatocyte growth factor (hepatopoietin A; scatter factor)	HGF	Hs.396530	NM_000601
HNF1 homeobox A	HNF1A	Hs.654455	NM_000545
Intercellular adhesion molecule 1	ICAMI	Hs.633447	NM_000201
Interferon, gamma	IFNG	Hs.856	NM_000619
Insulin-like growth factor 1 (somatomedin C)	IGF1	Hs.160562	NM_000618
Interleukin 10	IL10	Hs.193717	NM_000572
Interleukin 1, beta	IL1B	Hs.126256	NM_000576
Interleukin 6 (interferon, beta 2)	IL6	Hs.654458	NM_000600
Insulin-like growth factor 1 (somatomedin C)	INS	Hs.654579	NM_000207
Integrin, alpha 6	ITGA6	Hs.133397	NM_133397
Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen 51)	ITGAV	Hs.436873	NM_000210
Integrin, alpha X (complement component 3 receptor 4 submit)	ITGAX	Hs.248472	NM_000887
Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	ITGB1	Hs.643813	NM_002211
Jagged 1	JAG1	Hs.728907	NM_000214
Kinase insert domain receptor (a type III receptor tyrosine kinase)	KDR	Hs.479756	NM_002253
KIT ligant	KITLG	Hs.1048	NM_003994

Table 2
(*Continued*)

Description	Symbol	Unigene	Genbank
Leukemia inhibitory factor (cholinergic differentiation factor)	LIF	Hs.2250	NM_02309
Melanoma cell adhesion molecule	MCAM	Hs.599039	NM_006500
Microphtalmia-associated transcription factor	MITF	Hs.166011	NM_000248
Matrix metallopeptidase 2 (gelatinase A, 72 kDa gelatinase, 72 kDa type IV collagenase)	MMP2	Hs.513617	NM_004530
Nestin	NES	Hs.527971	NM_006617
Nerve growth factor receptor	NGFR	Hs.415768	NM_002507
Notch 1	NOTCH1	Hs.495473	NM_017617
Nudix (nucleoside diphosphite linked moiety X)-type motif 6	NUDT6	Hs.558459	NM_007083
K(lysine) acetyltransferase 2B	KAT2B	Hs.533055	NM_003884
Platelet-derived growth factor receptor, beta polypeptide	PDGFRB	Hs.509067	NM_002609
Phosphatidylinositol glycan anchor biosynthesis, class S	PIGS	Hs.462550	NM_033198
POU class 5 homeobox 1	POU5F1	Hs.249184	NM_002701
Peroxisome proliferator-activated receptor gamma	PPARG	Hs.162646	NM_015869
Prominin 1	PROM 1	Hs.614734	NM_006017
PTK2 protein tyrosine kinase 2	PTK2	Hs.395482	NM_005607
Protein tyrosine phosphatase, receptor type, C	PTPRC	Hs.644514	NM_002838
Ras homolog gene family, member A	RHOA	Hs.247077	NM_001664
Runt-related transcription factor 2	RUNX2	Hs.535845	NM_004348
Solute carrier family 17 (anion/sugar transporter), member 5	SLC17A5	Hs.597422	NM_012434
SMAD family member 4	SMAD4	Hs.75862	NM_005359
SMAD specific E3 ubiquitin protein ligase 1	SMURF1	Hs.189329	NM_020429
SMAD specific E3 ubiquitin protein ligase 2	SMURF2	Hs.705442	NM_022739
SRY (sex determining region Y)-box 2	SOX2	Hs.518438	NM_003106
SRY (sex determining region Y)-box 9	SOX9	Hs.647409	NM_000346
Thy-1 Cell Surface antigen	THY1	Hs.644697	NM_006288
T-box 5	TBX5	Hs.381715	NM_181486
Telomerase reverse transcriptase	TERT	Hs.492203	NM_198253
Transforming growth factor, beta 1	TGPB1	Hs.645227	NM_000660
Transforming growth factor, beta 3	TGFB3	Hs.592317	NM_003239
Tumor necrosis factor	TNF	Hs.241570	NM_000594
Vascular cell adhesion molecule 1	VCAM1	Hs.109225	NM_001078
Vascular endothelial growth factor A	VEGFA	Hs.73793	NM_003376
Vimentin	VIM	Hs.643813	NM_003380
Wingless-type MMTV integration site family, member 3A	WNT3A	Hs.336930	NM_033131
Zinc finger protein 42 homolog (mouse)	ZFP42	Hs.335787	NM_174900

3.4. Extracellular matrix (ECM) and adhesion molecules

Stem cell shapes serve their specialized functions. A round, spherical shape allows for maximal lipid storage in adipose tissue, while cell spreading facilitates osteoblast matrix deposition during bone remodeling [10]. During stem cell commitment, the evolution of cell morphology is arisen from changes in the expression of integrins, cadherins and cytoskeletal proteins. Undifferentiated stem cells express a wide variety of genes related to the extracellular matrix [11], as well as integrins. WJ derived stem cells expressed highly COL1A1 but also RHOA (cytoskeleton), MMP2 (extracellular matrix remodeling) and

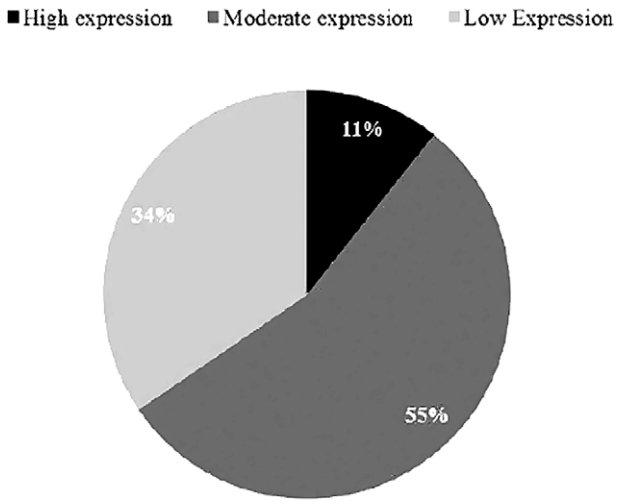

Fig. 2. Distribution of the expressed genes within three clusters according to their relative expression. Clusters were arbitrary fixed. Mean values used to validate the clusters model are representative of the number of donors within the cluster for all the genes whose relative expression's mean belongs to the cluster.

ANXA5. ITGAV, CTNNB1 and ITGA6 mRNA were expressed moderately whereas ITGAX mRNA were found weakly (Fig. 3(D)). These transcripts could explain the potential implication of stem cells in tissue remodeling, tissue repair and angiogenesis which were reported by other groups [12].

3.5. Trophic property

The trophic property of stem cells is mediated through the secretion of a broad spectrum of growth factors and cytokines that induce cell proliferation, angiogenesis and neurogenesis, influencing then their local microenvironment [12]. Endothelial cell recruitment/differentiation and vascularization are subsequent to BDNF, KDR, ICAM1, IGF1, EGF, PDGFRB, VCAM1 and INS release. mRNA corresponding to all of these molecules are moderately expressed by our WJ derived stem cells populations excepted for KDR, IGF1 and INS (Fig. 3(C) and (E)). In the same way, in addition to FGF, we found expression of others genes corresponding to mitogenic proteins as TGFB1 and 3 and HGF (Fig. 3(E)). The presence of these transcripts strengthens hypothesis that WJ derived stem cells could have important role in tissue repair and remodeling. Through their trophic bioactive molecules, bone marrow-derived MSCs support hematopoiesis *via* secretion of hematopoietic cytokines such as MGCSF, GCSF and IL-6 [13]. WJ-SCs expressed CSF2, CSF3 and NOTCH1 at low level but had a moderate expression of IL6 and JAG1 mRNA. WJ-derived stem cells could support hematopoiesis.

Via a paracrine effect of cytokines, it has been described that MSCs assist and modulate tissue regeneration *via* anti-inflammatory and immunomodulatory mechanisms [12]. mRNA of immunomodulatory cytokines as IL10, IL1B, TGFB1, TNF, INFG and cell adhesion molecules as ICAM1 have been analyzed. Our cells as reported previously expressed IL6 mRNA but also ICAM1, IL1B and TGFB1 mRNA at moderate level and TNF, IL10 and INFG mRNA at low level. Furthermore, an immunomodulatory effect of the corresponding proteins, prevents proliferation and function of many immune cells as T cells, Natural Killer cells, monocytes, macrophages.... Finally, all common mRNA genes corresponding to signaling pathways (SMAD, PKT2, PTPRC, GAPDH, ...) have been represented in Fig. 3(F). Most of these genes are moderately expressed.

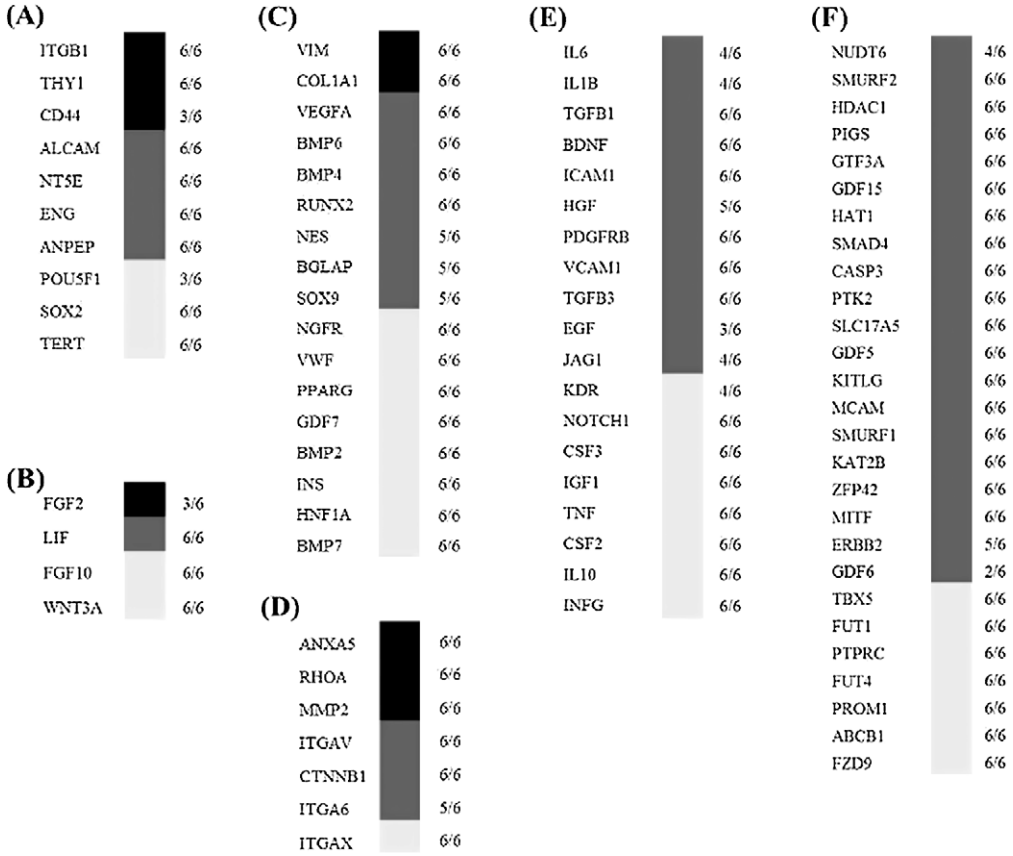

Fig. 3. Human mesenchymal stem cells RT2 profiler PCR array analysis of WJ derived stem cells. Black color, considered as high expression, represents transcripts with more than 16,384 copies above the threshold. Dark grey color, considered as moderate expression, represents genes with number of transcripts between 16,384 and 128. Grey color, considered as low expression, represents transcripts with less than 128 copies above the threshold. Fractions represent the number of donors for which the relative expression value is included within the indicated cluster. (A) Stem cell markers; (B) Self renewal and undifferentiated state genes; (C) Master genes of the three embryonic layers lineages; (D) ECM and adhesion molecules; (E) Trophic properties and immunomodulatory genes and (F) Other genes.

4. Conclusion

Combining these results, Wharton's jelly derived stem cells represent a hypothetical active factor in further regenerative therapy oriented-studies. They are an alternative for bone marrow-derived stem cell use in cell/tissue therapy and regenerative medicine. Although informative, the view provided by our data is still restricted and needs to be complemented with data from other approaches. Further prospective researches using proteomic assays will be required.

Acknowledgements

The authors are very grateful to the staff at the obstetric department of Reims maternity for providing umbilical cords. This work was partly supported by Ville de Reims and SFR-CAP SANTE.

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