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Interpreted gene expression of human dermal fibroblasts after adipo-, chondro- and osteogenic phenotype shifts

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ABSTRACT

Autologous cell-based therapies promise important developments for reconstructive surgery. *In vitro* expansion as well as differentiation strategies could provide a substantial benefit to cellular therapies. Human dermal fibroblasts, considered ubiquitous connective tissue cells, can be coaxed towards different cellular fates, are readily available and may altogether be a suitable cell source for tissue engineering strategies. Global gene expression analysis was performed to investigate the changes of the fibroblast phenotype after four-week inductions toward adipocytic, osteoblastic and chondrocytic lineages. Differential gene regulation, interpreted through Gene Set Enrichment Analysis, highlight important similarities and differences of induced fibroblasts compared to control cultures of human fibroblasts, adipocytes, osteoblasts and articular chondrocytes. Fibroblasts show an inherent degree of phenotype plasticity that can be controlled to obtain cells supportive of multiple tissue types.

1. Introduction

The requirement of cells, albeit alleviated by the use of biocompatible scaffolds, is often a limiting factor in tissue reconstruction. Research on adult stem cells has led to some substantiation of the hope of obtaining autologous tissue-specific cells through techniques involving cellular differentiation. In theory, an autologous stem cell approach can provide the necessary cells required for any tissue reconstruction while avoiding the problems associated with graft rejection and immunosuppressive treatment. So far, such methods have only been applied clinically in very limited fashion, involving only a few cell types and with varying degree of success (Lazarus et al., 1995; Horwitz et al., 1999; Krampera et al., 2007). Among the impediments to using adult stem cells are difficulties associated with their harvest and culture. More importantly, the validation of differentiated phenotypes required for quality control is problematic. A better understanding of the inherent variations in phenotypic states is motivated and we approach this by looking broadly at the expression profiles of fibroblasts, three differentiated fibroblast types and the three wild-type targets.

Fibroblasts (FB) can be obtained through small skin biopsies, greatly expanded using standardized procedures, and are routinely used clinically. Dermal FB may be induced to differentiate towards adipocytes (AC), osteoblasts (OB) and chondrocytes (CC) using defined induction media (Junker et al., 2010; Sommar et al., 2010; Toma et al., 2001; Bartsch et al., 2005; Lorenz et al., 2008). These features make FB appealing candidates for use in tissue engineering applications. Nonetheless, current biological classification of the FB cell type is still equivocal (Sorrell and Caplan, 2009). Cellular phenotypes are indirectly defined by their particular pattern of regulated gene expression, and differentiation involves switching from one pattern of gene expression to another (Ben-Tabou de-Leon and Davidson, 2007). Certain transcription factors included in our analysis, key regulating transcription factors, are required for the development of specific tissues and control phenotype-defining expression (Lander et al., 2001; Pei, 2009). Microarrays were used to measure global gene expression and the results further interpreted through gene-set enrichment analysis (GSEA). Gene Ontologies and pathways found through GSEA identify putative biological attributes of the induced phenotypes and we demonstrate a basis for a broad inherent phenotype plasticity of FB at the level of gene expression.

Abbreviations: A-FB, adipogenic media induced; FB AC, human adipocyte reference group; C-FB, chondrogenic media induced; FB CC, human chondrocyte reference group; FB, human dermal fibroblasts (control group); O-FB, osteogenic media induced; FB OB, human osteoblast reference group

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2. Materials and methods

2.1. Cell sources and reference cultures

Human dermal FB and preadipocytes were isolated from discarded tissue obtained from routine reduction abdomenoplasty. Skin was obtained from both men and women aged between 40 and 65 years of age without any reported skin disease. Tissues were processed within 2 hours of surgery and cell isolation was performed as previously described (Junker et al. 2010; Entenmann and Hauner 1996). FB cultures were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal calf serum (FCS), 50 U/ml penicillin and 50 μg/ml streptomycin (PEST). Preadipocytes were maintained in DMEM/Ham's F12 (1:1) with 10% FCS and PEST, until differentiated to adipocytes (AC) using an adipogenic medium (Pittenger et al., 1999).

Human OB were obtained from tissue discarded after total hip arthroplasty. Bone was minced and incubated for 45 min in 1 mg/ml collagenase (type 2, Invitrogen). Cells were expanded in the DMEM supplemented with 10% FCS, PEST, 1 μM dexamethasone, 50 μM A2P and 10 mM β-glycerophosphate (BGP) (Jaiswal et al., 1997). Human articular CC were isolated from the discarded tissue following total knee arthroplasty as described by Pettersson et al., 2009. Cultures were maintained in the DMEM supplemented with 10% FCS, PEST, 10 mM 2-[4-(2-hydroxyethyl)-piperazin-1-yl]ethanesulfonic acid (HEPES), 0.1 mM MEM non-essential amino acid solution, 0.4 mM L-proline, and 0.2 mM ascorbate-2-phosphate (A2P) (Johnstone et al., 1998).

Cells used in the described experiments were cultured in polystyrene cell culture flasks at 37 °C, 5% CO_2 and 95% humidity. Culture expansion at ratios between 1:3–1:5 were employed using an ethylene-diamine-tetraacetic acid (EDTA) (0.01%)/Trypsin (0.125%) solution at 37 °C for approximately 15 min at 70–90% confluency. Cells between passages P4 and P8 were included in the experiments. Human material was handled in accordance with ethical standards at the University Hospital of Linköping, Sweden.

2.2. Induction of fibroblast differentiation

The isolated FB were passaged at least three times before the experiments were started. This practice ensures that contaminating keratinocytes, microvascular endothelial cells and melanocytes are negligible in the cultures. Replicate FB cultures were subjected to induction media (Table 1) for four weeks with media changed every 48–72 h. Control FB cultures were maintained in parallel throughout the experiments. Time points for staining, immunohistochemistry (IHC) and semi-quantitative real-time reverse-transcriptase PCR (qRT-PCR) were one (TP1), two (TP2), three (TP3) and four (TP4) weeks of induction. All experiments were repeated three to eight times with cells from different donors. Induced FB are referred to as A-FB, C-FB and O-FB (Table 1).

2.3. Histology and immunohistochemistry

Initial analyses of induced cultures were performed according to previously published methods (Junker et al., 2010). Briefly, A-FB cultures were stained for intracellular lipid accumulation using Oil Red-O (#O0625, Sigma, Stockholm, Sweden) (Blanchette-Mackie et al., 1995) and analyzed using antibodies towards perilipin A (dilution 1:200; #P1998, Sigma), lipoprotein lipase (LPL) (dilution 1:100; #SAB1401231, Sigma) and peroxisome proliferator/activator gamma (PPARγ) (dilution 1:50; #AV32880, Sigma). C-FB were stained for glucoseaminoglycans with Alcian Blue stain (#A3157, Sigma) (Lev and Spicer, 1964; Mason, 1971) and analyzed using antibodies towards aggrecan (dilution 1:50; #AB3778, Abcam, UK), collagen II (dilution 1:100; #MAB8887, Abcam), and Sox9 (dilution 1:50; #HPA001758, Sigma). O-FB were stained for calcified matrix using von Kossa staining (Bills et al., 1974) and Alizarin Red (Dahl, 1952) (#A5533, Sigma) and analyzed using antibodies towards osteocalcin (dilution 1:500; #AB1857, Chemicon, CA) (Beresford et al., 1984), osteonectin (Malaval et al., 1994) (dilution 1:1000; #AB1858, Chemicon) and Runx2 (dilution 1:50; #WH0000860M1, Sigma). Primary antibodies were detected using AlexaFluor 488 (green) or 546 (red) conjugated secondary antibodies (dilution 1:500; Invitrogen). Cell nuclei were visualized using 4',6-diamidino-2-phenylindole (DAPI) either through sample mounting in ProLong Gold DAPI (Invitrogen), or using a solution of 300 nM DAPI (Invitrogen). The antibodies used in this study have previously been tested for cross-reactivity and specificity (Junker et al., 2010). Images were captured using BX41 (40X/0.75, 20X/0.50) or IX51 (20X/0.40) light/fluorescence microscopes (Olympus, Sweden) fitted with DP70 cameras and appropriate fluorescence excitation/emission filters. Controls included positive reference cultures and omission of primary antibodies.

2.4. RNA isolation

RNA was isolated from discrete replicate cultures, each sample containing between 0.5 and 8×10^6 cells at each TP. RNA was isolated from AC after preadipocyte differentiation, OB and CC after 14 days (TP2). Cells were detached by trypsin–EDTA treatment and washed in phosphate-buffered saline solution (PBS) (centrifuged at 200 g for 5 min). RNA was isolated using the NucleoSpin RNA II kit (Macherey-Nagel GmBH, Germany) according to manufacturer's protocol (rev. 10, 2009). RNA was eluted in 60 μl dH_2O and checked spectrophotometrically in a NanoDrop 2000 (Thermo Scientific, MA) to determine purity and yield. Initial samples were assessed by the Bioanalyzer 2100 (Agilent Technologies Inc., CA) and all samples had RNA integrity number above 9.0. Samples were stored at -70 °C for up to three months and re-measured with the NanoDrop 2000 before use in experiments.

2.5. Relative quantification with real-time PCR (qRT-PCR)

Reverse transcription was carried out using a High Capacity RNA-to-cDNA Synthesis Kit (Applied Biosystems, CA), according to manufacturer's protocol but modified linearly for 50 μl reactions. 1 μg RNA was reverse transcribed in each sample. Hydroxymethylbilane synthase (*HMBS*) was selected as endogenous control based on candidate reference gene testing (TaqMan Express

Table 1
Induction media used for the differentiation of fibroblast cultures. PEST=penicillin (50 U/ml) and streptomycin (50 μg/ml), DEX=dexamethasone, IBMX=isobutylmethylxanthine, A2P=ascorbate-2-phosphate, BGP=beta-glycerophosphate, and TGF-β_1=transforming growth factor beta 1.

Induction medium	Designation of induced group	Formulation	Reference
Adipogenic	A-FB	DMEM, 10% FCS, PEST,1 μM DEX, 0.5 mM IBMX, 10 μM insulin, 200 μM indomethacin	(Pittenger et al., 1999)
Osteogenic	O-FB	DMEM, 10% FCS, PEST,0.1 μM DEX, 50 μM A2P, 10 mM BGP	(Jaiswal et al., 1997)
Chondrogenic	C-FB	DMEM, 1% FCS, PEST,1.125 μM insulin, 50 nM A2P and 10 ng/ml TGF-β_1	(Johnstone et al., 1998)