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Yield and characterization of subcutaneous human adipose-derived stem cells by flow cytometric and adipogenic mRNA analyses

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Abstract

Background—Adipose-derived stromal/stem cells (ASC) capable of multipotential differentiation can be isolated with high yield from human subcutaneous lipoaspirates. This study reports our recent experience isolating and immunophenotypically characterizing ASCs from >60 human subjects of mean age 43.6 and mean body mass index of 27.

Methods—We examined the ASC yield per unit volume of lipoaspirate tissue, their surface antigen profile based on flow cytometry, their histochemical differentiation potential along the adipogenic and osteogenic pathways, and their expression of adipogenic mRNAs by transcriptomic microarray and RT-PCR.

Results—The population (n = 64) of predominantly Caucasian (84.3%) female (90.6%) donors had a mean age of 43.6 ± 11.1 years and a mean body mass index of 27.0 ± 3.8 . A yield of $375 \pm 142 \times 10^3$ ASC was obtained per ml of lipoaspirate within a 4.1 ± 0.7 day culture period (n = 62). The ASC population was uniformly CD29⁺ CD34⁺ CD44^{lo} CD45^{lo} CD73⁺ CD90⁺ CD105⁺ and capable of undergoing both adipogenesis and osteogenesis in vitro based on Oil Red O and Alizarin Red staining, respectively. Adipogenic differentiation was associated with the significant induction of multiple mRNAs associated with lipid storage and synthesis based microarray analysis of n = 3 donors. During an adipogenic differentiation time course, representative mRNAs (*adiponectin*, *C/EBP α* , *leptin*, *LPL*) displayed increases of several orders of magnitude.

Discussion—These findings demonstrate the reproducibility of subcutaneous lipoaspirates as a consistent and abundant source of functional ASCs from donors across a spectrum of ages and

BMI. These results have relevance to regenerative medical applications exploiting autologous or allogeneic ASCs for soft and hard tissue engineering.

Keywords

Adipogenesis; Adipose-derived stem cells; Cell yield; Differentiation; Flow cytometry; Human; Transcriptome

Introduction

Since their original identification by Friedenstein in bone marrow (1), mesenchymal stromal cells (MSC) have been found in multiple tissues (2–4). In contrast to bone marrow, placenta, umbilical cord, skeletal muscle, and other sites, adipose tissue offers advantages in terms of its accessibility, abundance, and regenerative capacity as well as the willingness of many individuals to consent to its donation for tissue engineering and regenerative medical applications (5–7). The potential utility of adipose-derived stromal/stem cells (ASCs) has been demonstrated in multiple pre-clinical animal models for orthopedic, soft tissue, and ischemic injury (6, 8–20). Similar to bone marrow MSCs, the ASCs have been characterized based on their immunophenotypic and differentiation properties (21–26). Because large volumes of adipose tissue can be obtained from individual donors, it is possible to obtain high yields of ASC within a single passage (21). The current manuscript reviews our experience in isolating and characterizing primary ASCs from lipoaspirates in a cohort of subjects of > 60 subjects over a two year period between July, 2007 and June, 2009. In addition to quantification of cell yields, in vitro differentiation, flow cytometric immunophenotype, and adipogenic mRNA data are presented.

Materials and Methods

Materials

All reagents were purchased from Fisher Scientific (Dallas, TX) or Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Donor Selection and Informed Consent

All protocols were reviewed and approved by the Pennington Biomedical Research Center Institutional Review Board prior to tissue collection. All tissue was obtained from patients undergoing elective liposuction surgery. A signed consent agreement was obtained by the plastic surgeon and tissues were provided to the investigators in an anonymous manner. The demographic data on each subject was limited to age, ethnicity, gender, height, weight, and history of metabolic illness.

Donor Demographics

Adipose tissue specimens were obtained with informed consent from subjects undergoing elective liposuction or abdominoplasty surgery (n = 64). The majority of subjects were women (female, 90.6%; male 4.7%, not recorded 4.7%). Ethnically, the majority of subjects were Caucasian (84.3%) while African Americans (9.4%), Asians (1.6%) and not recorded

(4.7%) comprised the rest of the individuals. Their ages ranged from 18 to 66 (mean 43.6 ± 11.1 years) and their body mass index (BMI) ranged from 18.3 to 37.2 (mean 27.0 ± 3.8).

Isolation and Culture of ASC

ASC were isolated from fresh human subcutaneous adipose lipoaspirate according to published methods with some minor modifications (21, 27). The lipoaspirate tissue was extensively washed with warm phosphate buffer solution to remove erythrocytes and then digested in PBS supplemented with 0.1% Collagenase of Type I (Worthington Biochemical Corporation), 1% BSA, and 2 mM CaCl_2 for one hour at 37°C . Following room temperature centrifugation at $300 \times g$ and resuspension in Stromal Medium (DMEM/Hams F-12 Medium supplemented with 10% FBS (Hyclone, Logan UT) and 1% antibiotic/antimycotic, the stromal vascular pellet was plated at a density of 35 ml of lipoaspirate digest per T175 flasks (0.2 ml per sq cm). After 24 hrs of incubation at 37°C , 5% CO_2 , the adherent cells were washed with warm PBS and maintained in Stromal Medium until 80–90% confluent. The adherent population was harvested by digestion with trypsin (0.05%)/EDTA (1 mM) at 37°C for five minutes, washed in Stromal Medium and replated at 5×10^3 ASC per sq cm ("Passage 1" or "P1") or used in flow cytometric analyses (below) or cryopreserved (28) for future use.

Flow Cytometry of ASC

Trypsin harvested ASCs were washed with PBS three times and aliquots of 10^5 cells were incubated with phycoerythrin conjugated monoclonal antibodies directed against CD29 (eBioscience Cat # 12-0297) CD34 (Becton Dickinson Cat # 348057), CD45 (eBioscience Cat # 12-0459), CD73 (BD Pharmingen Cat # 550257), CD90 (BD Pharmingen Cat # 55596), or IgG $_{1\kappa}$ control (BD Pharmingen Cat # 555749) or FITC conjugated monoclonal antibodies directed against CD44 (BD Cat # 348057) or IgG $_{1\kappa}$ control (BD Pharmingen Cat # 554679) for 20 min on ice before being washed with PBS supplemented with 1% BSA three times and fixed in 1% formaldehyde overnight at 4°C . For each sample, 10^5 events were collected on a Becton Dickinson FACScaliber flow cytometer using CELLQuest acquisition software (Becton Dickinson) and analyzed using Flow Jo software (Tree Star). This antibody panel was selected, in part, based on the ISCT position paper on the criteria for defining MSCs (29).

Adipogenic and Osteogenic Differentiation of ASC

Confluent cultures of ASC (P1) were induced with Adipogenic Differentiation Medium (DMEM/Hams F-12, 3% FBS, 1% antibiotic/antimycotic, 0.5 mM isobutylmethylxanthine, 33 μM biotin, 17 μM pantothenate, 5 μM rosiglitazone (AK Scientific, Mountain View, CA), 1 μM dexamethasone, 1 μM insulin) for three days before being converted to Adipocyte Maintenance Medium (identical to Adipogenic Differentiation Medium without isobutylmethylxanthine and rosiglitazone). Cells were maintained for 9 days before fixation and Oil Red O staining. In selected experiments, samples of uninduced and adipogenic induced ASC were harvested for total RNA isolation at days 0, 3, 6, and 9 of differentiation. Confluent cultures of ASCs were converted to Osteogenic Medium (DMEM/Hams F-12 or DMEM, 10% FBS, 1% antibiotic/antimycotic, 10 mM β -glycerophosphate, 50 $\mu\text{g}/\text{ml}$ sodium 2-phosphate ascorbate, 10^{-8} M dexamethasone) and maintained in culture for 9–12 days

with medium changes every third day. The cultures were rinsed three times with 150 mM NaCl, fixed in 70% ethanol, and stained with Alizarin Red.

Total RNA Isolation, RT-PCR Methods, and Primers

Total RNA was isolated from ASC cultures using TriReagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. The total RNA (2 µg) was incubated in a 20 µl volume with Moloney Murine Leukemia Virus Reverse Transcriptase, dNTP, and olig-dT. RT-PCR was performed using SybrGreen PCR Master Mix (Applied Biosystems) using an ABI 7900 Real Time PCR System with primers for the following genes of interest: Adiponectin (NM_004797) (for) GGCCGTGATGGCAGAGAT (rev) TTTCACCGATGTCTCCCTTAGG; CAAT/Enhancer Binding Protein α (C/EBPα) (NM_004364.2) (for) GGGTCTGAGACTCCCTTTCCTT (rev) CTCATTGGTCCCCCAGGAT; Leptin (NM_000230.1) (for) GGTGCAAGGCCCAAGAA (rev) ACATAGAAAAGATAGGGCCAAAGC; Lipoprotein Lipase (LPL) (NM_000237.2) (for) TCCGCGTGATTGCAGAGA (rev) CGCTCGTGGGAGCACTTC; and 18S RNA (NR_003286.1) (for) AAACGGCTACCACATCCAAG; (rev) CCTCCAATGGATCCTCGTTA with the following cycling conditions (95°C for 10 min, 40 cycles of 95°C for 15 sec, 60°C for 1 min).

Transcriptomic Analysis

Total RNA was isolated from ASCs obtained from three individual donors and maintained under uninduced or adipogenic induced culture conditions for 9 days following confluence. The RNA was assessed by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA), cDNA synthesized using a Superscript cDNA Synthesis kit (Invitrogen, Carlsbad, CA) and a T7-(dT)₂₄ primer. Biotinylated cRNA was transcribed in vitro using a GeneChip IVT Labeling kit (Affymetrix, Santa Clara, CA), purified using a GeneChip Sample Cleanup Module, incubated in fragmentation buffer (200 mM Tris-acetate pH 8.1, 500 mM potassium acetate, 150 mM magnesium acetate) at 94°C for 35 min, chilled on ice, and hybridized to a Human U133 Plus 2.0 Array of ~47,400 transcripts and variants, including 38,500 well-characterized genes. Arrays were incubated at 45°C for 16 hrs rotating at 60 rpm, washed, stained at 25°C for 10 min with 10 µg/ml streptavidin R phycoerythrin (Vector Laboratories, Burlingame, CA), stained at 25°C for 10 min (X2) with 3 µg/ml biotinylated goat anti-streptavidin antibody (Vector Laboratories), washed, and scanned using a GeneChip Scanner 3000. Pixel intensities were measured, expression signals were globally scaled to a target intensity value of 2500, and features extracted using GeneChip Operating Software v1.2 (Affymetrix). Data mining and statistical analyses were performed with Data Mining Tool v.3 (Affymetrix) algorithms. The absolute call (present, marginal, absent) of each gene expression, the direction of change, and the fold change were identified with the above software. The raw Affymetrix CHP data has been stored in the NCBI Gene Expression Omnibus (GEO) data base under accession numbers GSM458608 (Uninduced), GSM458609 (Adipogenic Induced), and GSE18391 (Attributes). Additional analyses were performed with GeneGo (St. Joseph MI) and Ingenuity (Redwood City, CA) software programs.

Statistical Methods

Values are presented as the mean $\pm$ S.D. Student t-test was used to evaluate significance ($p < 0.05$). Linear regression was used to determine the correlation coefficient between ASC yield and donor age or BMI. Experiments were performed a minimum of $n = 3$ times with the exception of the microarray analyses where a single evaluation was performed on total RNA from uninduced and adipogenic induced ASCs of $n = 3$ biological donors.

Results

ASC Yield and Growth Time

The isolated stromal vascular fraction cells were plated immediately upon isolation at a density of 0.2ml of processed lipoaspirate tissue per sq cm. The adherent cell population, termed “Adipose-derived Stem Cells” (ASCs), reached 80–90% confluence based on visual inspection within 4.1 ± 0.7 days ($n = 62$). Following trypsin digestion and harvest of the “Passage 0” ASCs, the mean yield achieved was $375 \pm 147 \times 10^3$ cells per ml of lipoaspirate tissue (Table 1). The yield displayed a positive correlation with both donor age ($r = 0.30$) and BMI ($r = 0.26$); however, since the SVF cell plating density at the initiation of passage was not assessed, the significance of these values is open to question.

Flow Cytometric Analyses

The immunophenotype of the ASCs at P0 was determined by flow cytometric analysis using a panel of monoclonal antibodies (Figure 1). The ASC displayed the following mean ($\pm$ S.D.) percentage positive cells ($n = 64$ donors) for the indicated surface antigens: CD29 (β_1 integrin) $99.0 \pm 1.7\%$; CD34 $90 \pm 9.5\%$; CD44 (hyaluronate receptor) $15.5 \pm 8.5\%$; CD45 (leukocyte common antigen) $12.0 \pm 6.0\%$; CD73 (5' ectonucleotidase) $86.2 \pm 7.2\%$; CD90 (Thy1) $90.0 \pm 6.0\%$; CD105 (endoglin) $97.8 \pm 1.5\%$. In contrast, ASC stained with isotype control antibodies conjugated to phycoerythrin or FITC displayed background fluorescent intensities of $6.1 \pm 3.2\%$ and $4.8 \pm 3.3\%$, respectively.

Differentiation Analysis

The isolated P0 ASCs routinely displayed adipogenic and osteogenic differentiation potential in vitro. Figure 2 displays a representative photomicrograph of ASCs maintained for 9 days under uninduced (Toluidine blue), adipogenic (Oil Red O), or osteogenic (Alizarin Red) conditions.

Transcriptomic Analysis of Genes Induced 2-Fold with Adipogenesis

Transcriptomic microarray comparison of total mRNA from ASCs ($n = 3$ donors) identified a set of 1962 probe sets induced by 2-fold under adipogenic relative to undifferentiated conditions (Supplement Table 1); the raw Affymetrix datasets have been entered into the NCBI GEO database. The provisional set of potential differentially expressed genes was created using a 2-fold selection threshold. Pathways analysis based on Fisher's exact test identified gene categories (such as canonic pathways, genes interconnected by regulation, molecular function, etc.) significantly over-represented in the provisional list. Discovered pathways were adjusted for potential False Discovery Rate (FDR) estimated by Benjamini-

Hochberg algorithm (30). Both over-representation and FDR tests were implemented within the pathway analysis software. Our interpretation of gene expression pattern was based on functional groups (pathways). This approach, unlike stringent selection of microarray probes, allowed for a better accounting of interactive genes which may not be statistically significant considered separately, but add strength to each other as a group. Pathway analysis has been proven effective for the analysis of metastasis biomarker association with cancer (31, 32) and for signal transduction relationships with infectious disease (32). Ingenuity Pathway Analysis (Ingenuity Systems Inc., Redwood City, CA) of the induced microarray dataset identified specific metabolic pathways that could be associated with between 16 to 32 individual genes. Among the general metabolic pathways detected were those associated with cancer and cell proliferation, immunity, metabolism (amino acid, lipid, carbohydrate), extracellular matrix formation, and cell assembly/structure (Table 1, Supplemental Figure 1). Analyses of protein classes determined that significant enrichment (p -value < 0.05) was observed for the categories of enzymes, ligands, and proteases (Table 2, Supplemental Figure 2). Among these were enzymes involved in fatty acid metabolism which were induced with adipogenesis (Supplemental Figure 3). While there was no significant enrichment for transcription factors as an overall class, a subset of 59 transcription factors were identified that displayed highly significant interactions with Z -scores > 2.8 (Supplement Table 2). Among these are factors that have been previously associated with adipogenesis including members of the nuclear hormone receptor family, PAS domain proteins, CCAAT/enhancer binding proteins (C/EBP), sterol receptor enhancer binding protein (SREBP), and signal transducers and activators of transcription (STAT). The latter transcription factors mediate signal transduction via the gp130 protein receptor for interleukin 6; adipogenic associated changes in this pathway are highlighted in Supplemental Figures 4 and 5.

The time dependent expression of representative adipogenic genes was determined by RT-PCR (Figure 3) ($n = 4$ donors, BMI 23.1 ± 1.4 , age 39 ± 6 yr). The adipogenic transcription factors C/EBP α , the adipokines adiponectin and leptin, and lipoprotein lipase (LPL) all displayed a time dependent increase with adipogenesis, increasing between 3 to 7 orders of magnitude over a 9 day period of induction; however, the greatest percentage of this increase occurred within the initial 3 day period. Further studies were conducted evaluating mRNA levels at day 3 of adipogenic conditions in ASCs isolated from female, Caucasian donors with lean (mean BMI 22.82 ± 1.19 , $n = 6$, range 21.63–24.93), overweight (mean BMI 27.53 ± 1.37 , $n = 6$, range 25.81–29.4), or obese (mean BMI 32.04 ± 2.53 , $n = 6$, range 30.65–37.15) body mass indices. The mean ages of these cohorts (lean 37 ± 9.6 yr; overweight 41 ± 7.7 yr; obese 42 ± 10.4 yr) were not significantly different ($p > 0.4$, paired 2-tailed t -test). The expression levels for adiponectin, aP2, LPL, and PPAR γ 2 were not significantly different between the lean, overweight, and obese cohorts (Figure 4).

Discussion

The current results demonstrate that lipoaspirate tissue from donors across a wide age range yields substantial numbers of ASC per unit volume which display a consistent immunophenotype. Based on the experience reported in > 60 subjects, approximately 75 million cells can be recovered from 200 ml of processed lipoaspirate within a 4 to 5 day

culture period. In light of the fact that many liposuction procedures remove liter volumes of tissue, it is feasible to produce upwards of 375 million ASC per donor in less than one week. Calculations by Muschler and Midura (33) estimate that a cubic centimeter of bone contains 70 million cells. Thus, the yield from adipose tissue will approach or exceed the quantities of stem cells that are required for clinical regenerative medical applications. Based on the current study, donor ages up to 66 do not present an obstacle to recovering sufficient cell yields. This is consistent with recent work by Zhu et al. which did not find a significant effect of donor age on the proliferation rates of ASCs isolated from a cohort of women ($n = 15$) between the ages of 20 to 58 (34). While Zhu et al. did not find any effect of donor age on adipogenic potential of the ASCs, advancing age significantly reduced their osteogenic potential (34). De Girolamo et al. (35) have reported similar age-dependent results with respect to osteogenesis in a $n = 26$ women. Gender may also play a role since ASC from male, as compared to female, donors display increased osteogenic potential in vitro (36). As seen with ASC, advancing donor age has been correlated with reduced osteogenic activity in human bone marrow derived MSCs by some (37), but not all (38, 39), studies. Consistent with this and in contrast to the work of Zhu et al. (34), Schipper et al. have reported age dependent differences in ASC adipogenic and proliferative capacity (40).

They found that ASCs isolated from multiple depots in young (25–30 year old) donors proliferated more rapidly and expressed higher levels of PPAR γ protein as compared to older (55–60 year old) donors (40). The current study suggests that while donor BMI was associated with increased ASC yields, it had no significant effect on the expression of representative mRNAs on day 3 of adipogenesis (Figure 4). These reports suggest that further investigation should be conducted relating the impact of age and BMI on ASC functionality.

With respect to immunophenotype, the predominance of the current findings confirm and extend the existing literature. As expected of an MSC (29), the ASCs isolated from > 60 subjects consistently express high levels of CD29, CD73, CD90, and CD105 and low levels of CD45. However, in contrast to bone marrow MSCs (29), early passage ASCs have been shown here and by multiple other reports to be CD34 $^{+}$ (17, 18, 20, 22, 23, 41). It has been established that the expression of CD34 by early passage ASC is transient and decreases significantly with successive expansion to levels comparable to those found on BMSC (42). Thus, the timing of the flow cytometry measurements relative to the culture period is a critical determinant of the CD34 expression level. It remains to be determined if CD34 constitutes a stem cell marker for the ASC lineage.

One unexpected finding in the current report was the low level of CD44 expression on ASCs. Past analyses by our group (22, 23) and others (24) have detected CD44 on $>60\%$ of ASCs using the same monoclonal (L178) or related fluorochrome conjugated monoclonal antibodies. The detection of CD44 on $\sim 16\%$ of the ASC P0 population is puzzling. Since the CD44 gene is composed of 10 exons (43), it is possible that unique ASC splice variants are expressed and future work will explore the use of alternative epitope specific anti-CD44 monoclonal antibodies for immunophenotyping of the cells.

The ASCs isolated from multiple donors displayed the ability to undergo both adipogenic and osteogenic differentiation in vitro. Under adipogenic conditions, the ASC mRNA expression profile based on a microarray analysis identified a subset of genes and metabolic pathways that were significantly induced (Table 1, Supplement Table 1). These included multiple genes related to lipid synthesis and storage that have been detected previously in related studies of adipogenesis in human and murine cell models (44, 45). The RT-PCR analysis of four mRNAs on the list confirmed their robust induction under adipogenic conditions (Figure 3). In contrast, the undifferentiated ASCs expressed significantly higher levels of mRNAs associated with the extracellular matrix and Wnt signaling pathways (Supplement Table 1), consistent with published reports that have demonstrated adipogenic inhibition through Wnt mechanisms (44, 45).

In conclusion, these findings document the ability to obtain consistent yields of relatively homogeneous ASCs from human subcutaneous lipoaspirates harvested from donors across a wide range of ages and BMI based on flow cytometric immunophenotype and in vitro differentiation potential. These results have relevance with respect to the use of autologous and allogeneic ASC for regenerative medical applications in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Flow Cytometric Histograms. The fluorescence activated cell sorting analysis of human subcutaneous ASCs isolated from a representative donor are displayed using antibodies directed against CD29, CD34, CD44, CD45, CD73, CD90, and CD105 (solid line); all are conjugated to PE except for CD44 (FITC). Negative controls conjugated to FITC or PE are displayed to the left of each panel (dotted line).

Stromal

Adipogenic

Osteogenic

Figure 2.

Histochemical Differentiation. The human subcutaneous ASCs isolated from a representative donor were cultured to confluence and then maintained under control conditions in the presence of Stromal Medium (left panel), adipogenic conditions (middle panel), or osteogenic conditions (right panel) for 9 days. At the conclusion of these culture periods, the cells were fixed and stained with Toluidine Blue (control), Oil Red O (adipogenic), or Alizarin Red (osteogenic). Photomicrographs ($\times 20$ magnification) of the wells taken at $\times 20$ magnification (top) and scans of the entire well (bottom) are presented.

Figure 3.

Adipogenic Time Course by RT-PCR of Selected mRNAs. Human subcutaneous ASCs isolated from $n = 4$ donors were cultured to confluence and induced to undergo adipogenesis at day "0". Individual wells for each donor were harvested at times 0, 3, 6, and 9 after induction. RT-PCR was performed for the following adipogenic genes: *Adiponectin*, *CAAT/Enhancer Binding Protein α* (*C/EBPα*), *Leptin*, *Lipoprotein Lipase* (*LPL*). The levels of each were normalized relative to that of *18S RNA*.

Figure 4.

Adipogenic Induction of mRNAs in Donors of Varied BMI. Human subcutaneous ASCs isolated from lean (BMI < 25), overweight (BMI 25 to 30), or obese (BMI > 30) female, Caucasian donors (n = 6 per cohort) were induced to undergo adipogenesis and total RNA harvested on day 3. RT-PCR was performed for the following genes: *Adiponectin*, *aP2*, *Lipoprotein Lipase (LPL)*, *Peroxisome Proliferator Activated Receptor γ 2 (PPAR γ 2)*. The levels of each were normalized relative to that of *18S RNA*. Statistical comparisons were conducted with 2-tailed paired student t-test.

Table 1

Ingenuity Analysis of Transcriptomic Analysis of mRNAs Induced 2-Fold Under Adipogenic Conditions

Metabolic Pathway	Number of Times Identified
Cancer	16
Immune, Imflammatory, Infection, Hematopoietic	9
Development /Morphology	9
Lipid Metabolism	8
Fibrosis/Connective Tissue/Dermatology	3
Gastrointestinal Disease	3
Amino Acid Metabolism	2
Cell Assembly	2
Carbohydrate Metabolism	1

Table 2

Enrichment by Protein Class

Protein Class	r	R	P value	Z Score
Enzymes	261	2659	1.52E-09	6.245
Ligands	51	525	0.009621	2.527
Proteases	49	547	0.0404	1.876
Kinases	51	603	0.08348	1.482
Receptors	89	1431	0.1421	-1.122
Transcription Factors	65	951	0.4775	-0.1387
Phosphatase	15	226	0.4939	-0.184
Other	822	13255	7.79E-09	-5.755

Values are based on a total of 1403 network objects in the activated dataset and a total of 20197 objects in the complete dataset. The value “r” is the number of network objects in the activated dataset that interact with a chosen object while “R” is the number of network objects in the complete dataset that interact with the chosen object.