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Na^+, K^+-ATPase genes are down-regulated during adipose stem cell differentiation

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1. ABSTRACT

The expression of Na^+, K^+-ATPase alpha and beta subunits isoforms, FXYD2 and FXYD7 were studied in rat adipose stem cell (ASC) by qRT-PCR and immunofluorescence. ASCs were able to differentiate to chondrocytes or adipocytes. All studied genes were expressed in freshly isolated ASCs and in all passages checked. Immunostaining for alpha1 isoform was found in plasma membrane and nuclear envelope, alpha2 signal was lower and alpha3 staining was variable among cells. Beta isoforms signal was abundant and displayed an isoform-specific picture. Staining for FXYD7 was homogeneous in plasma membrane and cytosol. Chondrocytes differenciated from ASC showed identical Na^+, K^+-ATPase subunits isoforms expression patterns to chondrocytes in cartilage. The expression pattern of Na^+, K^+-ATPase genes in ASCs exhibits a unique phenotypic signature that implies functional differences in Na^+ and K^+ transport rates. Furthermore, this phenotypic signature may also be used as a complementary marker for studies of mesenchymal stem cell differentiation. We propose a possible 'moonlighting' role of Na^+, K^+-ATPase beta isoforms that could be essential for the study of mesenchymal stem cell function and differentiation.

2. INTRODUCTION

Mesenchymal stem cells derived from adipose tissue are undifferentiated pluripotent cells with the capacity to differentiate into a variety of specialized cell types (1). The differentiation of mesenchymal stem cells involves dynamic changes in gene expression which alters protein function, cell fate and phenotype commitment; a number of genes are up-regulated whereas others are down-regulated during the mesenchymal differentiation process (2-5). The expression pattern of genes encoding members of the Na^+, K^+-ATPase protein family varies among different cells and tissues and also during development (6-8). Specifically, chondrocytes express alpha1-3 and beta1-3 subunits isoforms (9). This transport system is embedded in the plasma membrane and is responsible for maintaining the intracellular Na^+ and K^+ concentrations characteristic of most animal cells. The enzyme consists of two principal subunits, alpha and beta and a non-obligatory FXYD protein. The three subunits exist as multiple isoforms, which can serve as markers of cell differentiation and phenotype (6-7). The alpha subunit also known as the catalytic subunit contains binding sites for Na^+, K^+, and Mg^{2+}. Furthermore it serves as the receptor for cardiac glycosides such as ouabain, a class of antiarrhythmic drugs

used for the treatment of congestive heart failure and cardiac arrhythmia. The beta subunit is a 45-55 kDa glycoprotein that modulates the transport properties of Na^+, K^+-ATPase (6-7). In addition the beta subunit acts as a molecular chaperone, regulating the structural and functional maturation of alpha subunits as well as controlling the formation of tight junctions and establishing epithelial cell polarity. FXYD proteins confer to the Na^+, K^+-ATPase specific modulatory requirements of different cells and tissues (i.e. variations in ion affinities of Na^+, K^+-ATPase subunits, ion-specific conductance, induction to ion channelling) (10).

Four different isoform variants have been described for the alpha subunit (alpha 1-4) (11). Of the four known alpha isoform genes, alpha1 appears to be ubiquitously expressed in all cells and tissues tested so far (7-8;11-12) whereas the alpha2 and alpha3 isoforms are expressed primarily in electrically excitable tissues; alpha2 has been found mainly in the brain, heart and skeletal muscle (8); alpha 3 is the most tissue-restricted in its expression, being found primarily in neurons in the brain (8) and in human and primate myocardium (13); alpha4 is expressed only in testis (11). The beta subunit is a highly glycosylated protein that interacts with the alpha-subunit and is involved in ion recognition (14) and structural and functional maturation. Four different isoforms of the beta subunit (beta1-3 and beta-m) have been described in vertebrates (15-17). beta1 is expressed in most cells and developmental stages, also there is evidence for a role in the polarized distribution of Na^+, K^+-ATPase based on the observation that beta1–beta1 linkage between adjacent cells contributes to cell–cell adhesiveness and polarized localization of Na^+, K^+-ATPase of epithelial cells (18-19). The beta2 isoform was independently identified as an adhesion molecule on glial cells (AMOG), specifically involved in neuron-astrocyte adhesion (16;20). The beta3 subunit (15) is expressed in brain, among many other tissues (15;21-24). In the nervous system, only retinal photoreceptors (25) and oligodendrocytes (24) have been shown to express the beta3 isoform at the protein level. beta-m plays a role in vertebrate evolution. In fish, amphibian and avian species, beta-m behaves as any other Na^+, K^+-ATPase beta isoform but in mammals, accumulates only in nuclear membrane of perinatal myocytes, has not known function and works as co-regulator of gene expression.

The FXYD subunit belongs to a gene family of ion transport regulators that contain the sequence FXYD and 7 invariant and 6 highly conserved amino acids (26). The FXYD proteins have been proposed to function as novel tissue- and isoform-specific regulators of Na^+, K^+-ATPase (27). Four of the FXYD proteins (FXYD1, FXYD2, FXYD4, FXYD7) have been shown to alter the activity of the Na^+, K^+-ATPase (28). FXYD2 corresponds to the γ isoform, the predominant renal FXYD isoform, and FXYD7 is the neuronal specific isoform.

alpha and beta subunit association is promiscuous, and all of the alphabeta subunit combinations may exist. However, the patterns of subcellular and developmental specification are extremely complex. In the system, both, the control of biosynthesis and the intrinsic enzyme properties are affected by the choice of alpha and beta isoform (6;8;29-30). The alpha subunit isoforms possess different kinetic properties and modes of regulation, and the beta subunit isoforms affect the ion affinities, expression and plasma membrane targeting of Na^+, K^+-ATPase isozymes. The expression pattern of genes encoding members of the Na+, K+-ATPase protein family varies among different cells and tissue and very little is known about the origins of Na+, K+-ATPase in stem cells and the spatiotemporal patterns associated with the expression of the respective genes. The presentation of the importance of this transport system becomes clear. Consequentially scientific efforts are directed towards the analysis of the physiologically required isozyme combinations.

Therefore, it is important to identify new stem cell markers with the capacity to provide additional information about the unique physiology and phenotype of these cells. As mentioned above, a large body of evidence has been collected over the last twenty years regarding the expression patterns of Na^+, K^+-ATPase isoforms in different cells and tissues. Despite this, very little is known about the origins of Na^+, K^+-ATPase in stem cells and the spatiotemporal patterns associated with the expression of its turning on and off of the gene family. In this study we have characterized Na^+, K^+-ATPase in freshly isolated adipose stem cells monitor changes in the genes encoding its isoforms in three generations (passages). In this paper we demonstrate that acutely isolated ASCs express all known Na^+, K^+-ATPase isoforms and, consequently, we deduce that during the differentiation process some of these genes are turned off to specify the required isozyme combinations required for the physiological function of the terminally differentiated cell types arising from mesenchymal stem cells.

3. MATERIALS AND METHODS

3.1. Isolation and culture of rat adipose mesenchymal stem cells

For isolation of adipose-derived stem cells *(*ASC), we used three moth old Sprague-Dawley rats, 250-300 g. (La Laguna University Animal Care Unit). Experimental procedures in rats were in accordance with the guidelines of the Animal Care Advisory Committee of the University of La Laguna. A total of 90 animals were used. After intraperitoneal anaesthesia (1µl/g Ketamine (40 mg/ml, Imalgene 1000, Merial) and Xilasine (4mg/ml, Rompun 2%, Bayer)) sacrifice of the rat was done by cervical dislocation, subcutaneous adipose tissue from inguinal zone was extracted and ASCs isolated using a modification of published methods of methods originally described by Rodbell and colleagues (31-32). Briefly, the adipose tissue, from 300 to 500 mg, was mechanically dissociated into pieces of about $1mm^3$, washed twice with Hank's Balanced Salts, without Ca^{2+} neither Mg^{2+} (HBSS, H2387, Sigma) for 10 min at 1200 rpm, followed by enzymatic digestion with collagenase 0,45% (Type IA, C2674, Sigma), in a 1:5 proportion with HBSS for 30 mins

at 37ºC with gentle shaking. After this, collagenase activity was inactivated by adding an identical volume of D50 (Dulbecco's modified Eagle medium, BE12.741F, Lonza and 10% Fetal Bovine Serum, DE14-802F, Lonza y 1% Pen/Step Amphotericin B 100x (10.000 U Pen/ml, 10.000µg Strep/ml y 25µg Amphotericin B/ml; 17-745E, Lonza)) and centriged 10 min at 1200 rpm. In order to eliminate remaining blood cells, the pellet was resuspended in 4 mL of D50 y and added to a Ficoll gradient, and centrifuged 2000 rpm/30 min. The intermediate layer was harvested, and washed twice with D50. Cell viability was assessed by trypan blue exclusion assay trypan blue 0,4%; T8154, Sigma). The cells were plated at a density of 3 x 106 cells/ 25 cm2 flasks and cultured in D50 at 37ºC with 5% CO2. After 24 h, the non-adherent cells were removed by rinse with HBSS (Hank's BSS with calcium and magnesium, 10-508F, Lonza) and the adherent ASC cells were maintain in D50 until 80% confluent and different passages fueron hechos (P1-P3). For each passage the cells were plated at a density of 3 x 10^5cells/ 75 cm^2 flasks. The ASC cultures were harvested by triypsin digestion.

All experiments were performed in triplicate using separate cultures from separate rats.

ASC cells were grown under either control conditions or with IGF-I (Sigma-Aldrich Chemie GmbH, Stenheim, Germany) supplemented media in a humidified environment at 37 °C with 5% CO_2 95% air. For all experiments described cells from passages 1-3 were used.

3.2. Differentiation of adipose derived mesenchymal stem cell

To show the multipotent differentiation potential of ASC (Dominici, M. 2006), induction to adipocytes and condrocytes was performed in P3 cultures in 12-well culture well chambered coverglass, 600/well. For adipogenic differentiation we use "StemPro® Adipogenesis Differentiation Kit" (Gibco, Invitrogen) as directed for the maker. The medium was changed every 3 days and test performed at 3 and 4 weeks, in 10 different samples. Samples were then processed for detecting lipid vacuoles by Oil Red O staining following standard protocols (Lenka Janderova's protocol for Thermo Fisher, SC protocol sheet:11) (33). Briefly, condrogenic induction medium was eliminated and cells washed in PBS, fixed in PFA 4% 30 min and Oil Red O stained. For chondrogenic differentiation and staining we used "STEMPRO® Chondrogenesis Differentiation Kit" (Invitrogen) proceeding as directed for the manufacturer at two different times (2 and 3 weeks culture) and stained with Alcian Blue.

3.3. Reverse transcription-linked polymerase chain reaction, real-time quantitative RT-PCR (qRT-PCR)

Total RNA was isolated from ASCs cultures at passage 1, 2 and 3 using SC Total RNA Isolatiom System (Promega, Z31000) according to the manufacturer's specification. Total RNA was reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad, 170-8890). Real-Time Rt-PCR was perfrmed on diluted cDNA samples with IQ SYBR Green Supermix (Bio-Rad, 179-8880) using the MiniOpticon Two-Color-Real Time PCR Detection System (Bio-Rad), under universal cycling conditions (95°C for 10 min, 40 cycles of 95°C for 20 sec, then 72°C for 1 min). Primers for Na^+, K^+-ATPase alpha, beta and subunits isoforms, FXYD2 and 7 modulator proteins and stem cell markers Thy1/CD90 and CD44 are specified in Table 1 (2). The RT-PCR for all primers pairs had been validated and determined to display single peaks in their dissociation curves. Melting curves analysis was performed to validate specific amplicon amplification without genomic DNA contamination.

Reactions were performed in triplicate, and multiple water blanks were included with the analysis. The house keeping gen, beta-actin, was used as a reference for mRNA quantification. Relative expression levels for each gene were normalized by the Ct value of the house keeping gene and gene expression was presented as arbitrary units of the ratio of Ct of the gene over Ct of beta-actin.

3.4. Antibodies

A panel of well-established isoform specific antibodies was used in this study, see table 2 for further details (21;34-36).

3.5. Immunofluorescence

For immunofluorescence, ASCs were plated on microscope glass slides or coverslips. Cells were rinsed with Dulbecco's PBS (DPBS), fixed with pre-chilled (–20ºC) 100% methanol for 6 min at –20ºC, washed with DPBS and stored overnight at 4ºC. Cells were permeabilized with 0.03% Triton X-100 and 0.1% BSA in DPBS for 30 min at room temperature, and incubated with primary antibodies in the same buffer for 1 h, washed 3 times, incubated in fluorescein- or rhodamine-labeled secondary antibodies in the dark for 1 h, washed extensively in DPBS, mounted in PBS:glycerol (1:9, v:v) and examined with an Olympus BX50 fluorescence microscope.

To demonstrate the specificity of our primary antibodies in immunofluorescence procedures, we carried out control experiments without either primary or secondary antibody and used positive control tissues for the respective proteins Na^+, K^+-ATPase (data not shown).

4. RESULTS

4.1. Control of ASC as stem cells and adipogenic and chondrogenic differentiation of ASC

Table 3 shows the values of positive expression of stem cell markers Thy1/CD90 and CD44 as well as lacking of expression of CD45 and CD34 markers at passages 1, 2 and 3 relative to beta-actin (1). Also markers CD29 and CD105 were positive (data not shown).In order to check the purity in ASC of our cultures we performed adipogenic and chondrogenic differentiation assays. Used kits droved ASC to a 90-100% adipocyte or chondrocyte cells (Figure 1).Both controls demonstrated that we used a rich ASC preparation.

Table 1. Primers and annealing temperatures used for qRT-PCR

Gene	Primer	Sequence	T annealing
ß Actin	RAT_AB-F	CCAACCGTGAAAAGATGACC	*
	RAT_AB-R	TACGACCAGAGGCATACAGG	
Alpha1	RAT_A1-F	GGACAAACTTGTGAACGAGC	59ºC
	RAT_A1-R	CTCCACATCATTGATCCAGC	
Alpha 2	RAT_A2-F	ACGGATCAGCTGAACTTTCC	59ºC
	RAT_A2-R	CACCATGATCACCTTGATGC	
Alpha 3	RAT_A3-F	AGATCGTCTTTGCCCGAACC	59ºC
	RAT_A3-R	GACATCAGAGCCAGCAATGC	
Alpha 4	RAT_A4-F	TGATTGCACCCATGAGAACC	60ºC
	RAT_A4-R	GTCTTGCCCATTGTCAATCC	
Beta 1	RAT_B1-F	GTCCTAATGACCCCAAGAGCTACG	60ºC
	RAT_B1-R	GTGGGCATACTGCCACAATCC	
Beta 2	RAT_B2-F	CTGACCATACCCCCAAGTACC	60ºC
	RAT_B2-R	CTTGGATGGAGTCGTTGTAAGG	
Beta 3	RAT_B3-F	GAAGAAATCCTTCCACCAGAGC	60ºC
	RAT_B3-R	GAGCAAGATGAGACCCCAGC	
FXYD7	RAT_FXD-F	TGCCTCAGCATTCAGCAACC	61ºC
	RAT_FXD-R	CCCAGCACGAACATGATAGTGG	
FXYD2	RAT_Fxyd2-F	TATGACTATGAAACCGTCC	54ºC
	RAT_Fxyd2-R	GCCTATGCTTCTTACTGC	
Thy-1	RAT_Thy-1-F	ACTCTAGCCAACTTCACCACC	59ºC
	RAT_Thy-1-R	TTATGCCACCACACTTGACC	
CD44	RAT_CD44-F	GTCTTGCCCATTGTCAATCC	59ºC
	RAT_CD44-R	TGAGTCACAGTGTCGAAACC	

* The annealing temperature for beta-actin is identical to that of Na^+, K^+-ATPase isoform or stem cell marker in each qRT-PCR reaction.

Table 2. Antibodies used for the detection of Na^+, K^+-ATPase subunit isoforms

Antibody	Target	Species	Dilution	Type	Source
α6F	α1 isoform of Na^+, K^+-ATPase	Mouse	1:4*	Monoclonal	DSHB**
McB2	α2 isoform of Na^+, K^+-ATPase	Mouse	1:4*	Monoclonal	K.J. Sweadner (34)
α3	α3 isoform of Na^+, K^+-ATPase	Rabbit	1:600*	Polyclonal	M. Caplan
SpETβ1	β1 isoform of Na^+, K^+-ATPase	Rabbit	1:600	Polyclonal	P. Martín-Vasallo (36)
SpETβ2	β2 isoform of Na^+, K^+-ATPase	Rabbit	1:600	Polyclonal	P. Martín-Vasallo (36)
RNTbeta3	β3 isoform of Na^+, K^+-ATPase	Rabbit	1:150	Polyclonal	K.J. Sweadner (21)
FXYD7	FXYD7 isoform of Na^+, K^+-ATPase	Rabbit	1:600	Polyclonal	K. Geering (35)

* Antibody-antigen complexes were detected using the Biotin-Streptavidin technique ** Developmental Studies Hybridoma Bank, University of Iowa - The 6F hybridoma developed by Douglas Fambrough was obtained from the DSHB developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242.

4.2. Expression levels

The relative expression levels of the different isoforms of the plasma membrane Na^+, K^+-ATPase subunits were studied in ASCs using qRT-PCR. From the time the cells were isolated to the harvest of the third passage cells, the total time taken was from 18 to 21 days. Cell isolation to confluence for first passage usually took 10 to 12 days, from this passage to second and from second to confluence took 4 to 5 day each, Figure 2. Table 3 shows the values of Na^+, K^+-ATPase alpha, beta and subunits isoforms, FXYD2 and 7 modulator proteins and stem cell markers Thy1/CD90 and CD44 at passages 1, 2 and 3 relative to beta-actin. All the genes were expressed in the four stages studied, although alpha2 and alpha3 showed variability in their levels. This point will be further explained along with the expression at the protein level.

4.3. Cellular localization

The cellular distribution of Na^+, K^+-ATPase alpha and beta subunit isoforms and FXYD2 and 7 was assessed in freshly isolated ASCs and cells from passages 1, 2 and 3 using a panel of isoform-specific antibodies. Figure 3 shows the distribution of Na^+, K^+-ATPase alpha subunit isoforms in cells. Staining for alpha1 isoform was present all along the studied period; it was localized in the plasma membrane of ASC cells at the highest expression level, compared to other isoforms, Figure 3. In freshly isolated ASC it was present in the nuclear envelope and showed a punctuated pattern that evolved to a homogeneous distribution. Alpha2-isoform specific immunoreactivity was present in plasma membrane of ASCs at a lower level, but always considerable regarding the mRNA levels. This divergence could be explained by considering than the recycling pool of protein is enough as for giving immunofluorescence signal although there is no concomitant synthesis at that moment. No appreciable signal for alpha3 isoform appeared in most cells, only a few isolated cells showed immunofluorescence limited to plasma membrane in not all preparations examined, Figure 3. Cellularity was established by staining cell nuclei with propidium iodide. Because of the apparent contradictions between previous results of "*in situ*" hybridization for alpha3 subunit and immunofluorescence data for this isoform, we used two different and very well characterized antibodies, the monoclonal XVIF9G10 and the polyclonal alpha3 (Table 2). To test further the apparent contradiction with our results we performed a series of duplicated samples in which we fixed the tissue in 4%

Table 3. Mean and standard deviation of Na^+, K^+-ATPase isoforms and Thy-1 and CD44 stem cells markers relative gene expression and CT ($\bar{x}$) obtained by qRT-PCR in ASCs at passages 1, 2 and 3

PASS	GENE	CT(x)	RELATIVE EXPRESSION
1	$alpha_1$	22.37	39.59 ± 15.08
	$alpha_2$	32.91	0.20 ± 0.19
	$alpha_3$	34.67	0.0 ± 0.0
	$alpha_4$	NA	NA
	beta1	28.38	11.05 ± 5.75
	beta2	32.87	0.14 ± 0.02
	beta3	25.54	23.70 ± 6.35
	FXYD2	29.16	0.60 ± 0.43
	FXYD7	32.19	0.77 ± 0.71
	Thy-1	21.19	262.51 ± 2.48
	CD-44	27.42	4.59 ± 2.48
2	$alpha_1$	23.19	71.49 ± 19.83
	$alpha_2$	NA	NA
	$alpha_3$	34.60	0.08 ± 0.05
	$alpha_4$	NA	NA
	beta1	31.48	0.44 ±0.17
	beta2	34.28	0.07 ± 0.03
	beta3	25.71	52.14 ± 33.93
	FXYD2	27.16	3.69 ± 1.89
	FXYD7	34.79	0.59 ± 0.57
	Thy-1	22.12	357.63 ± 46.57
	CD-44	28.37	12.91 ± 8.05
3	$alpha_1$	23.73	35.88 ± 10.51
	$alpha_2$	NA	NA
	$alpha_3$	33.89	0.05 ± 0.03
	$alpha_4$	NA	NA
	beta1	31.66	1.15 ± 0.76
	beta2	33.94	0.19 ± 0.11
	beta3	24.54	94.75 ± 34.73
	FXYD2	31.20	0.58 ± 0.08
	FXYD7	34.92	0.59 ± 0.51
	Thy-1	20.12	422.67 ± 174.10
	CD-44	27.91	2.46 ± 0.75

Means are from triplicates of a minimum of four cases. NA, no amplification.

paraformaldehyde: results were the same as those described before in fresh-frozen sections (see Materials and Methods section).

Regarding the beta subunit of Na^+, K^+-ATPase, specific immunoreactivity for Na^+, K^+-ATPase beta subunit isoforms is shown in Figure 4 for all passages studied. Staining for beta1 isoform forms a bright nuclear envelope surrounded by a lower reactivity in the cytoplasm and plasma membrane of ASCs. High beta2 immunoreactivity was seen in nuclear envelope; the fluorescence signal was less bright in periphery of freshly isolated ASCs, then, the signal decreased in intensity all along the cytosol until the plasma membrane that delineates the periphery of the cell. Specific immunoreactivity for Na^+,K^+-ATPase beta3 subunit isoform is shown Figure 4. beta3 subunit isoform was present in the plasma membrane, soma and nuclear envelope of ASC, the signal adopted the form of 'crispy sparks' as if they formed 'clusters' grouped in 'rafts'. This type of signal was sharper in freshly isolated ASCs; total immunofluorescence intensity increased with passages, as far as the period studied in this investigation, and the signal became more homogeneous, although maintaining a 'spark like' pattern in the background.

FXYD7 antibody showed a homogeneous immunofluorescence signal in ASC with an even distribution in plasma membrane and cytoplasm, characteristic of all images are the positive labelling of what seems to be nucleoli of ASCs, Figure 5.

4.4. Lack of IGF-I effects on Na^+, K^+-ATPase expression in ASCs

No significant effects were found in mRNA expression of any Na^+, K^+-ATPase isoform after treatment of ASCs, either fresh or cultured with IGF-I within the limits of this study.

4.5. Na^+, K^+-ATPase expression in chondrocytes differentiated from ASCs

We checked by qRT-PCR and immunohistochemistry all isoforms in chondrocytes differentiated from ASC. Cells showed identical Na^+, K^+-ATPase subunits isoforms expression patterns to chondrocytes in cartilage (9), at both, mRNA and protein levels, that is Na^+, K^+-ATPase alpha1-3 and beta1-3 subunits isoforms.

5. DISCUSSION

In this study we present new experimental data to support the idea that freshly isolated ASCs express/contain alpha1, alpha2 and alpha3, beta1, beta2 and beta3 and FXYD2 and FXYD7. Although these cells lack the testis-specific alpha4 isoform they still possess the capacity to form several protomeric isozyme

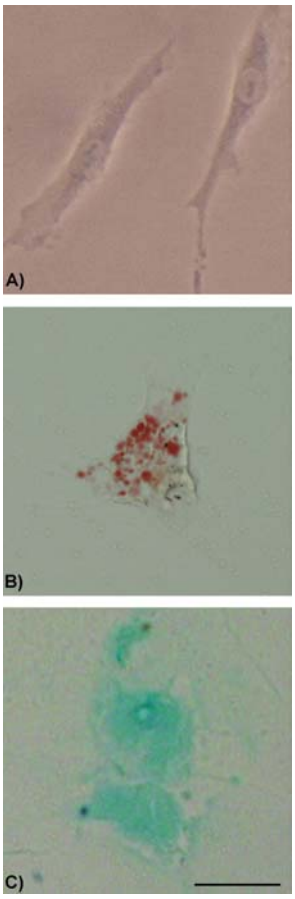

Figure 1. Adipocyte (Panel B) and Chondrocyte (Panel C), differentiated rat ASCs. Panel A, phase contrast image of rat ASC. Scale bar 100 μm.

combinations. Our data suggests that the alpha1beta3 combination is the most abundant isozyme. The alpha1 isoform was found to be continuously expressed throughout this study, and, so far, no cell has been described lacking this isoform. The beta and FXYD isoforms are also continuously expressed in ASCs. At the first passage expression of the alpha3 isoform and synthesis of the alpha3-mRNA stops abruptly and the expression of the alpha2 isoform is also reduced at the mRNA levels. However, both isoforms are still present at the protein level albeit at lower levels of expression. At the second and final passage state, we found no alpha2-mRNA and alpha3 was expressed at low levels. The expression of alpha subunits in the four culture points here described clearly differentiate ASCs from adipocytes, which are known to express the alpha1 and alpha2 isoforms (37). The identity of the beta subunit in differentiated adipocytes is not known. The beta1 isoform is expressed in pre-adipocytes (undifferentiated 3T3 cells) but when these cells are induced to differentiate beta1 expression disappears and beta2 is not detected at all. Furthermore, with no extrinsic growth factors added, ASCs express alpha3 and all beta isoforms, a pattern that is distinctly different from that of differentiated adipocytes (38-40). FXYD2 and FXYD7 are kidney and brain-specific isoforms respectively; they have not been found in adipocytes thus far (41).

At the present time we can confidently state that all the antibodies selected for use in this study are well established. There are no reports of any cross-reactivity with other proteins and other Na^+, K^+-ATPase isoforms (42;43). More importantly for the purposes of this study, antibodies to beta1, beta2 and beta3 do not cross-react with beta-m. The apparent inconsistencies between our qRT-PCR and immunofluorescence data may be due to the de-synchronization between mRNA synthesis and protein turnover or different translational efficiencies in different passages. This could also be the result of cell harvesting effects.

The morphological evaluation of beta isoform localization in freshly isolated ASCs presents a distinguishable picture: a) beta1, discontinuously outlined cariolemma, homogeneous cytosol and light plasmalemma; b) beta2, sharp-fluorescent nuclei, lesser intensity peri-nuclear envelope, lighter peripheral cytosol and sharp-discontinuous plasma membrane; c) beta3, homogenous brilliant-dotted nuclei surrounded by a darker ring limited by a pencil drawn thick and discontinuous nuclear envelope within a light background full of fluorescent linear sparks, as an impressionist painting. Furthermore, depicted pictures agree with the established model of cotraslational insertion of beta isoforms in the endoplasmic reticulum and periphery of the nuclear envelope (44-45).

5.1. Kinetic differences of Na^+, K^+-ATPase isozymes in ASCs

Isoforms of the alpha subunit confer significantly different kinetic properties to isozymes. The beta-isoform associated influences the Na^+ (14) and K^+ affinities (46-47) of any formed isozyme, although not as much as the alpha-polypeptides do. Apparent affinities for Na^+, K^+ and ouabain have been determined in recombinant enzymes generated in heterologous expression systems, such as insect Sf-9 cells (6) or *Xenopus laevis* oocytes (29;48). Na^+, K^+-ATPase isozymes of the rat formed in Sf-9 cells present a relative Na^+ affinity as follows: alpha2beta2>alpha2beta1>alpha1beta1=alpha3beta2>alpha3beta1 and that for K^+, alpha1beta1>alpha2beta1=alpha2beta2>alpha3beta1=alpha3beta2 (6). Affinities of human isozymes expressed in *Xenopus laevis* oocytes are alpha1beta1>alpha2beta1>alpha3beta1 for Na^+ and alpha3beta1=alpha1beta1>alpha1beta3>alpha1beta2>alpha2beta1>alpha3beta3>alpha3beta2>alpha2beta3>alpha2beta2 for K^+ (29). Studies mentioned before also show differences in the kinetic parameters and enzymatic behavior of isozymes. These discrepancies among species and expression systems led to uncertainties in the functional characteristics of the isozymes possibly formed during differentiation and, possibly, influenced by different affinities during the promiscuous subunit association. In addition to these differences, the kinetics become more complex when the FXYD subunit enters the working system and modulates the tissue-specific mechanisms of Na^+, K^+-ATPase function (49), more specifically, FXYD2

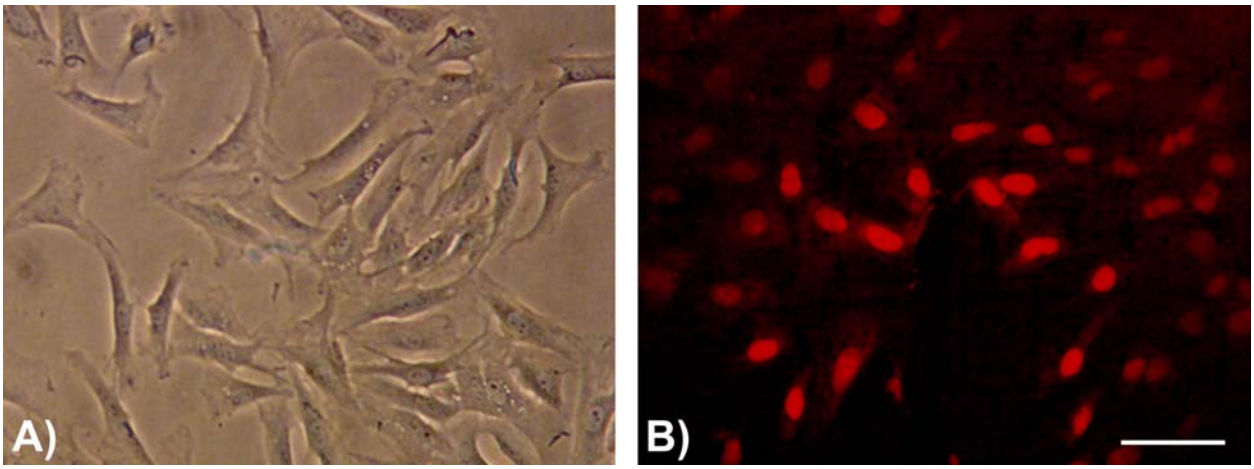

Figure 2. Characterization of rat ASCs. Rat ASCs from 1-3 passages were used for our experiments. Panel A, phase contrast image of rat ASC mono-layers from passage 0 to passage 3. The cells exhibit a large and flat morphology under phase contrast. Panel B, Nuclei are labeled with propidium iodide (red). Scale bar 100 μm.

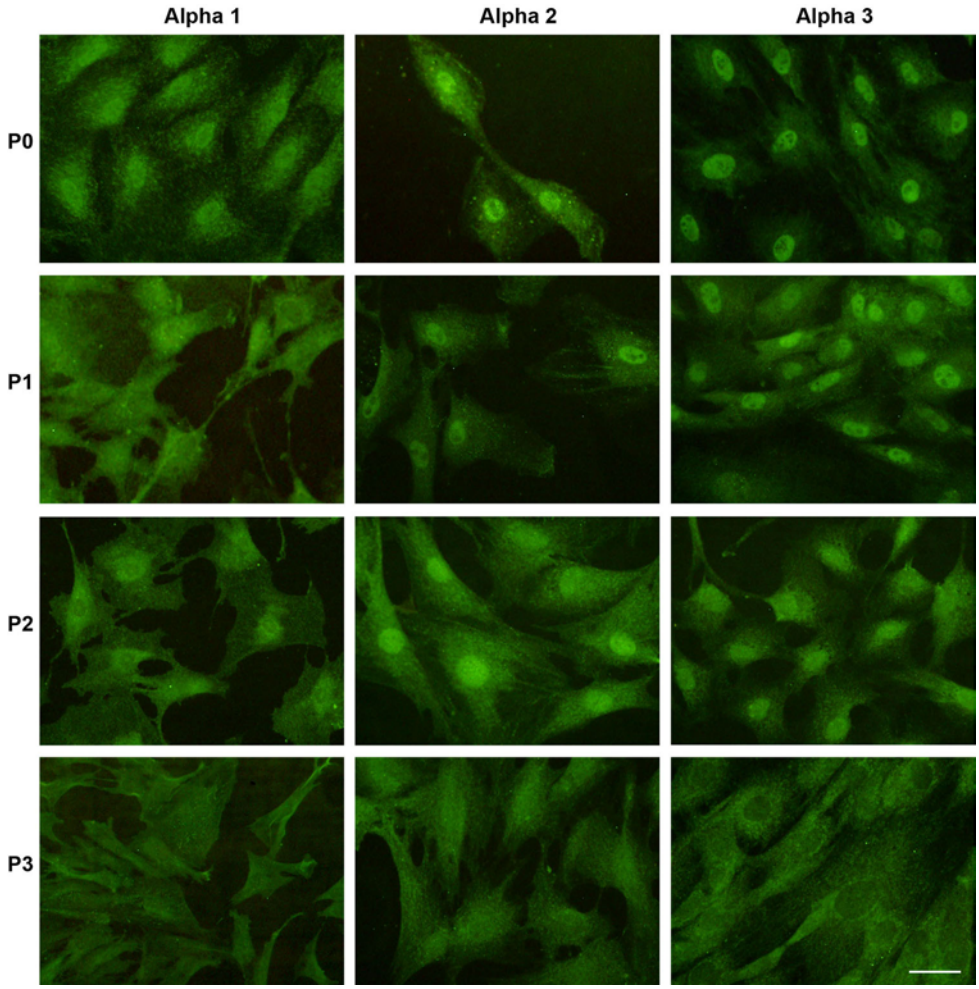

Figure 3. Immunofluorescence images of ASCs labelled with anti-alpha subunit isoforms specific antibodies. Immunolocalization of alpha1, alpha2 and alpha3 isoforms in freshly isolated cells (P0) and cells from passages 1 to 3 (P1-3). Scale bar 50 μm.

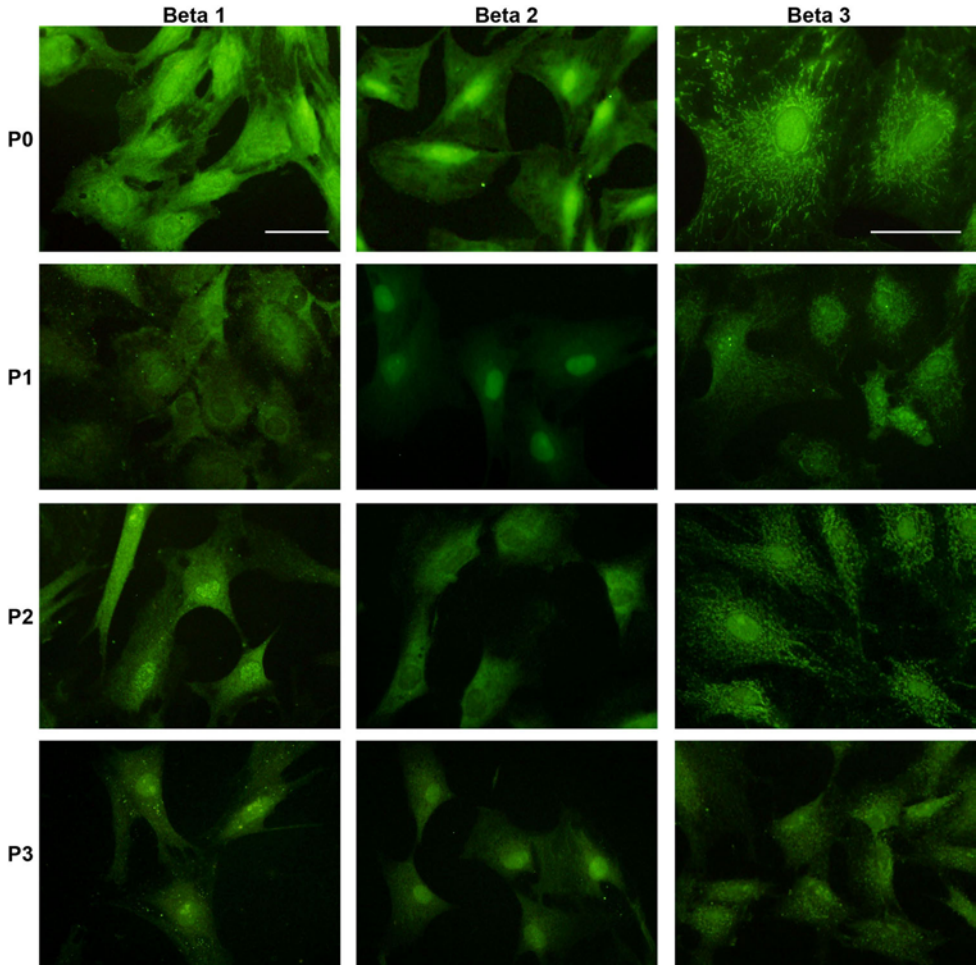

Figure 4. Immunofluorescence images of ASCs labelled with anti-beta subunit isoforms specific antibodies. Immunostaining for beta1, beta2 and beta3 isoforms in freshly isolated cells (P0) and cells from passages 1 to 3 (P1-3). Scale bar 50 μm for all panels but P0beta3, that is larger.

decreases the apparent sodium affinity and FXYD7 decreases the apparent potassium affinity of Na^+, K^+-ATPase. Based on our data, we conclude that ASCs are able to handle Na^+ and K^+ with all possible affinities and may reflect a critical role of Na^+, K^+-ATPase in cell survival and/or adaptation to ionic hypertonic stress, in addition, given the uneven distribution of isoforms, isozymes formed create microenvironments specific for other more complex functions as adhesion or polarization, facts more specific for beta subunits that will be commented in next section.

5.2. Comments on other specific roles of the beta subunit

The lack of alpha2-mRNA, low alpha3-mRNA expression levels and only alpha1 mRNA and protein normal expression associated to much higher levels of all three beta isoforms drove us to consider other potential moonlighting roles (50-52) of the beta subunits in ASCs. Moonlighting proteins perform different functions in different subcellular locations or at different developmental or evolutionary stages. Three moonlighting roles agree with the localization described for the beta subunits: molecular chaperone activity, protecting against degradation during routing to membrane integration and functional protomer formation of the newly synthesized catalytic alpha subunit (10;41). Three more roles, in a complex manner, are associated with the alpha subunit biogenesis process and Na^+, K^+-ATPase activity; beta subunit has a role in the formation of tight junctions, in suppression of cell motility and cell polarity in epithelial cells through interactions of tight junctions with membrane channels and transporters (18;53-55). Thus, it seems like all beta isoforms resemble beta-m subunits of mammals, which have lost their transporting function and placed in the cariolemma function as co-regulators of gene expression (17). FXYD2 and FXYD7, which will become kidney and brain specific isoforms are both expressed in ASCs (10).

5.3. The lack of IGF-I effects on Na^+, K^+-ATPase in ASCs

Based on the opposite effects of IGF-I in two different systems - bovine embryos that develop to the

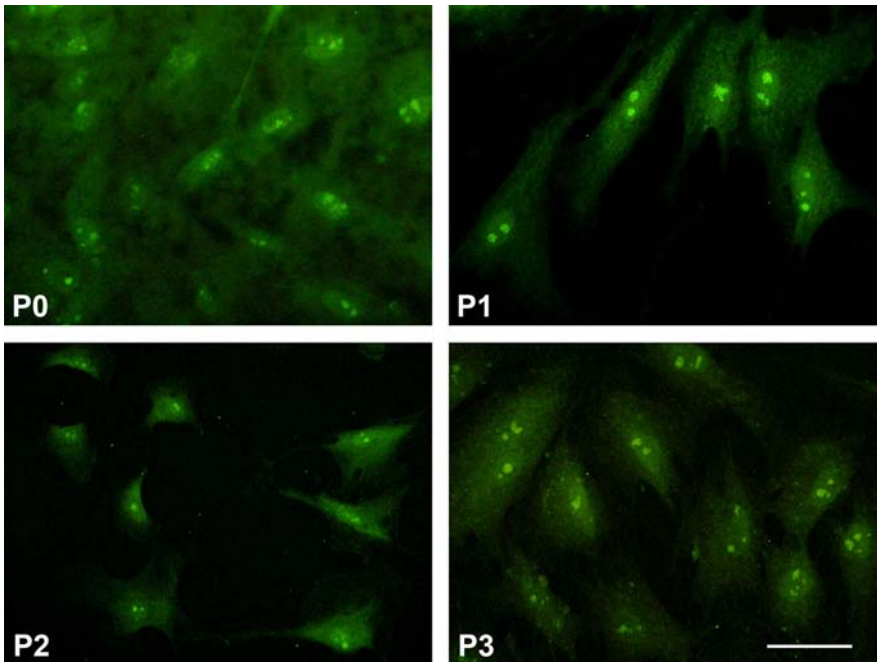

Figure 5. Immunofluorescence images of ASCs labelled with anti FXYD7 specific antibodies; freshly isolated cells (P0) and cells from passages 1 to 3 (P1-3). Scale bar 50 μm.

blastocyst stage, IGF-I increased mRNA for Na^+, K^+-ATPase and other genes (56) and, on the contrary, in gills of the fish striped bass, IGF-I decreased by 50% levels of alpha-subunit Na^+, K^+-ATPase mRNA after 24 h control incubation (57), we set out to determine the effect of IGF-I on Na^+, K^+-ATPase in ASCs in this study. No differences were found using our culture system and the data obtained was not statistically significant (data not shown). This indicates that IGF-I effects may be exerted much later than the ASC stage, perhaps at a primordial tissue stage (i.e. in the blastocyst), or in specific tissues in well-defined conditions or described variations in Na^+, K^+-ATPase enzymatic activity, if any, as due to cellular pool redistribution.

In conclusion, the characteristic and cell-specific expression pattern of Na^+, K^+-ATPase subunit isoforms may be used as a complementary marker during mesenchymal stem cell differentiation or when ASCs are intended for research or therapeutic purposes. Interestingly, the typical isoform distribution within the cell suggests that the beta isoforms may fulfil additional 'moonlighting' roles and functions, for example maintaining the cellular shape and, probably along with beta-m, as co-regulators of gene transcription.

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