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Pancreatic Reg I and Acinar Cell Differentiation: Influence On Cellular Lineage

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Abstract

Objectives—Pancreatic reg I has been implicated in cellular differentiation. Acinar cells can transdifferentiate into other pancreatic-derived cells, and we postulated that changes in intracellular levels of reg I would affect the state of differentiation.

Methods—We transfected AR42J cells with a plasmid containing the entire coding sequence of reg I, and isolated clones with cDNA in sense (SS) or antisense (AS) orientation. Levels of mRNA and protein expression were examined by Western blotting and Real Time-PCR.

Results—Expression of reg I was confirmed in sense or antisense clones. AR42J transfected with SS demonstrated more acinar-like phenotype while those transfected with AS showed a less differentiated state. Specifically, amylase mRNA and protein levels increased in SS cells while AS cells showed increased PDX-1 and insulin mRNAs and cytokeratin protein. Conversely, cytokeratin and PDX-1 were depressed in SS cells.

Conclusions—These data demonstrate that in acinar cells, reg I over-expression is linked to acinar cell differentiation, while inhibition of reg I leads to beta-cell and possibly ductal phenotype. Reg I expression in acinar cells is important in maintaining pancreatic cell lineage, and when decreased, cells can de-differentiate and move towards becoming other pancreatic cells.

Keywords

pancreatic reg I; acinar cells; AR42J; sense overexpression; antisense inhibition

INTRODUCTION

The pancreatic reg (pancreatic regenerating) gene family is comprised of several isoforms that have been isolated from normal and regenerating pancreas [1]. In the rat, reg I mRNA and protein are constitutively expressed in pancreatic acinar cells but not in islets or ductal epithelia. Its overexpression is associated with pancreatic regeneration and islet formation [2,3]. While we and others have shown it to be mitogenic to ductal and beta cells [4,5] its induction during pancreatic injury suggests it may have a role in cellular differentiation.

We and others have studied reg I levels during cellular differentiation [6–8], and the results are conflicting. While some studies indicate that the levels of reg I mRNA and protein inversely correlate with cellular differentiation [6,7,9], other studies show no correlation [8]. Since all these studies were done on fixed tissue or cultured cells where reg I was induced with agents which can confound results (eg. dexamethasone), we wished to test the effect of reg I gene expression alone on the morphology on the acinar cell.

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Acinar cells have been recently shown to be able to transdifferentiate into islet-like tissue [10] or duct-like tissue [11]. We postulated that changes in pancreatic reg I expression within the acinar cells is involved in phenotype changes. To study this potential effect, we transfected the cell line AR42J with a constitutively expressed vector containing either sense and antisense clones of rat reg I, and documented the acinar, ductal and endocrine properties of the cell.

METHODS

We used the amphicrine rat acinar pancreatic cell line AR42J (ATCC, Rockville, MD), which normally expresses reg I mRNA and protein [3]. AR42J cells have exocrine and neuroendocrine properties and secrete amylase, elastase and other digestive enzymes. [12,13]

Reverse-transcription polymerase chain reaction (RT-PCR) of the coding region of rat reg I cDNA was generated by reverse transcription of total RNA extracted from pancreata taken either from normal rats by incubation with avian myeloblastosis virus reverse-transcriptase under standard reaction conditions. Primers for polymerase chain reaction (PCR) were chosen based on the full coding sequence of the published rat Reg I mRNA as follows: sense [1–23] AGCCTGCAGAGATTGTTGACTTG and antisense [628–651] AGGGGGTTGACTTTGCTTTTGATA [14]. This yielded a predicted product of 651 bp encompassing the coding region of the whole protein. PCR was conducted as follows: 5 minute denaturation at 95°C; 30 cycles of annealing (30 seconds at 62°C), extension (30 seconds at 72°C), and denaturation (30 seconds at 95°C), followed by a final extension step (7 minutes at 72°C).

The PCR fragments were ligated into the pCR®3.1 vector (Eucaryotic TA Expression Kit bidirectional, Invitrogen, Carlsbad, CA). This vector contains the entire coding sequence driven by a CMV promoter. We then isolated clones with cDNA in sense (SS) or antisense (AS) orientation; DNA sequencing, to confirm correct reading frame and orientation, was performed on a Applied Biosystems 373 sequencer at the SUNY Downstate Medical Center DNA Sequencing Core Facility using dye terminator chemistry.

AR42J cells were transfected with sense, antisense or empty pCR 3.1 vector plasmid DNA using LipofectAMINE PLUS reagent (Gibco BRL, Grand Island, NY) at 50 % cell confluency according the manufacturer's instructions in medium containing 1 μg of construct. Stable transfected cell lines expressing sense, antisense rat reg I or empty vector were selected using G418 (Invitrogen, 800μg/mL).

The transfected cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum, 100U/ml penicillin, 100ug/ml streptomycin and 200 μg/ml G 418 and incubated in a humidified incubator at 37°C with 95% air and 5% CO_2.

Two sense, two antisense and two empty vector clones were chosen for experiments, and each experiment was repeated at least 3 times.

Western blotting

Proteins isolated from Amicon (Beverly, MA) concentrated supernatants (10 ug total) were subjected to 12% SDS-PAGE. The proteins were transferred to a nitrocellulose membrane for immunodetection with three respective antibodies: Murine anti-reg I at 1:1000 (prepared in our laboratory), Pan anti-cytokeratin mixture at 1:50 (C9687 Sigma, St. Louis, MO) and rabbit anti-amylase polyclonal antibody at 1:1000 (gift from Dr. Catherine Figarella, Marseille, France). The membranes were incubated with either horseradish peroxidase-conjugated anti-rabbit (1:2000) or anti-mouse (1:5000) IgG antibody (Amersham, Pharmacia Biotech). The

membranes were developed with enhanced chemiluminescence Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ), stripped,a nd reblotted with b-actin to confirm loading. The intensity of the bands was analyzed by densitometry with ScanImage software.

Amylase assay

The quantity of amylase was quantitated from 10 ug aliquots of cell lysates using a chromogenic method using Vitros AMYL microslide test (Johnson & Johnson Clinical Diagnostics, Inc, Piscataway, NJ).

Cell Growth—MTS-tetrazoleum assay (CellTiter 96, Promega, Inc., Madison, WI) was described previously [14]. Colorimetric assay was performed on an absorbance microplate reader (Bio-Rad, Inc., Hercules, CA) at 490 nm. Each experiment was done in triplicate and repeated at least twice.

Real-Time quantitative PCR by SYBR green detection

Reverse transcription of total RNA treated with DNase I was performed using the Omniscript Reverse-Transcriptase (Qiagen, Valencia, CA). The primers (sense and antisense) used in the PCR reaction were deduced from the nucleotide sequences, and are shown in Table 1.

Quantitative real-time PCR was performed on a GeneAmp® 5700 Sequence Detection System apparatus (PE, Applied Biosystems, Foster City, CA). The cDNA synthesis was carried out in a 20 μl reaction volume containing 1 μg DNase I-treated total RNA. The amplifications were carried out in a 96 well plate in a final volume of 50 μl containing 2 μl (for β-Actin and amylase) or 4 μl (for Pdx1 and Insulin) of cDNA sample, 0.25 μM each of primers and 1× QuantiTect ™ SYBR® Green PCR mix (Qiagen). After 15 min at 95°C to denature the cDNA, the cycling conditions were as follows: 40 cycles consisting of denaturation at 94°C for 30 s, annealing at 63°C for 30 s, and extension at 72°C for 30 s. Each sample had 2–3 replicates. The average C_T value for target mRNA was subtracted from the average β-actin C_T value to yield the Δ C_T value. The average Δ C_T value for the control (expression of stable cell line following transfection with empty vector) was then subtracted from the Δ C_T value of each sample to give the ΔΔ C_T. The fold change in expression level was calculated using the formula $2^{-(\Delta\Delta CT)}$.

Statistical analysis—Values are expressed as mean ± SD; comparisons were made by the Student t test and were considered significant at $p < 0.05$.

RESULTS

Immunodetection of reg I protein on AR42J transfected with reg I antisense (RegAS) and reg sense (RegS) clones

Two clones were chosen for experiments - two empty vector controls, two reg I antisense (RegAS2 and RegAS10), and two reg I sense vectors (RegS2 and RegS3). As mentioned in Materials and Methods, orientation was confirmed by DNA sequencing (not shown). Western blot analysis revealed a reduction in immunoreactivity of reg I protein in antisense clones and an enhancement in immunoreactivity in the sense clones, when compared to controls (Fig 1A). Densitometric analysis revealed that when compared to empty vector controls, intensities of bands were reduced by over 50% for the antisense clones (54± 1.8% for RegAS2 and 65± 8.2% for RegAS10, $p < 0.01$) and increased by over 60% for the sense clones (161± 5.9% for RegS2 and 182± 24% for RegS3, $p < 0.01$).

Changes in reg I expression induce morphological differentiation in AR42J cells and affect cell growth

The morphology of the AR42J cells changed in response to transfection with reg I cDNA antisense or sense orientation (Fig 2). Antisense cell lines RegAS2 and RegAS10 exhibited more spheroidal morphology, consistent with a state of less differentiation (Fig 2B). In contrast, reg I sense lines RegS2 and RegS3 have a flattened morphology appearance with fewer clusters, increased intracellular vacuoles and exhibit the initial stages of organization into a reticular formation (Fig 2C). These changes of differentiation towards an acinar state are very similar to that which we have observed previously [7].

Figure 3 shows that both sense and antisense transfectants exhibited lower growth rates than empty vector plasmid transfectants alone; there was no difference between the latter and native AR42J cells (not shown).

Changes in reg I expression affect the cellular levels of amylase

We measured amylase using both Western analysis (Fig. 4) and real time PCR (Table 1, to be discussed below). No consistent change in amylase content was observed in antisense cell lines, but in the sense-transfected cells amylase levels increased. Figure 4 shows that levels of amylase in the cloned RegS2 and RegS3 cell lines were two-fold higher than cells transfected with empty vector (2.00 ± 0.20 and 2.2 ± 0.3 U/mg, respectively, compared with vector level of 1.13 ± 0.13 U/mg). This change is also similar to what we have seen before with AR42J differentiation [7]. No differences were noted between empty vector and native AR42J (not shown).

Changes in reg I expression alter the expression of cellular cytokeratins

While ductal cells express large amounts of CK19, AR42J cells normally express small amounts of cytokeratins (CK8 and 18), and endocrine cells do not express either [15,16]. Cytokeratins are therefore good markers for differentiation of AR42J. Figure 5A shows Western analysis of a panel of cytokeratins in antisense and sense clones compared to empty-vector transfected cells. Antisense reg I cells increased cytokeratin expression by over 30%, and sense clones showed reduced levels by at least 20%. Figure 5B shows cytokeratin proteins were higher in RegAS2 and RegAS10 by 134 ±9.3% and 136± 13.8%, respectively ($p < 0.001$). By contrast, protein levels were reduced in RegS2 and RegS3 clones by 84±12% and 65±11%, respectively ($p<0.01$).

Changes in reg I expression induce changes of expression of amylase, Pdx1 and insulin in AR42J

Amylase, Pdx-1 and insulin gene expression were measured by quantitative real-time PCR (Table 2). As noted in the Western analysis, amylase protein levels in sense-transfected clones were significantly elevated when compared to empty-vector transfectants, while no consistent change was noted in the antisense clones.

Pdx-1 is an important homeobox gene in the development of the pancreas; it is part of the upstream regulation of expression of islet cell-specific gene products such as insulin. When compared to empty vector controls, transfection of AR42J with reg I antisense significantly increased both Pdx-1 and insulin expression, whereas transfection with reg I sense resulted in no consistant change in Pdx-1 or insulin expression.

DISCUSSION

Recently the plasticity of the pancreatic acinar cell has been established. Acinar cells can differentiate into islets[11], ducts [10] and even pancreatic cancer [17]. Pancreatic reg I has

been implicated in acinar and epithelial cellular differentiation, but it has not been well studied. While it is an established mitogen of beta and ductal cells, reg I mRNA has also been found to be ectopically expressed in areas of the gastrointestinal tract during times of de-differentiation, specifically in colon and rectal tumors[6] and stomach [18]. In particular, we found reg I to be highly expressed in two areas of the gastrointestinal tract where differentiation is important- the 'transition zones' around colon cancers [9], and in Paneth cells in patients with ulcerative colitis [19].Reg I gene expression may be involved in gastrointestinal tumorigenesis; some have proposed it to be a marker of differentiation of gastrointestinal epithelia [3,9].

In this study we focused on the role of reg I in the pancreas. To determine if changes in cellular expression of reg I will alter the acinar cell's state of differentiation, we manipulated reg I gene expression in the rat acinar cell AR42J. We transfected the cell line AR42J with a vector which constitutively expressed rat reg I in either a sense or antisense orientation, and documented the response of AR42J to these manipulations using acinar, ductal and endocrine markers. We showed that the antisense reg I vector did in fact depress reg I protein expression, and resulted in a more undifferentiated state by microscopy, with induction of a endocrine phenotype as exhibited by expression of, Pdx-1 and insulin genes, and possibly a ductal phenotype by increased cytokeratin. By comparison, we proved that transfection with a sense reg I vector resulted in increased reg I protein expression, a more differentiated acinar state in culture, less cytokeratin than controls and higher amylase protein and mRNA levels.

Therefore, we succeeded in manipulating reg I gene expression directly inside the acinar cell without subjecting the cell to external stimuli such as drugs or disease - this allowed us to study the true effect of reg I gene expression on acinar cells. Our findings show that manipulation of reg I can affect cellular differentiation of the amphicrine AR42J acinar cell line: increased reg I expression directs the cells to a more differentiated 'acinar' state, while antisense reg I directs the cells to de-differentiate and move towards an endocrine or possibly ductal pathway.

AR42J does not express the reg I receptor, which is a transmembrane protein [20], and is likely the modulator of mitogenesis [4]. So, despite the fact that reg I is an established mitogen to beta and ductal cells [4,5,16], it is not surprising that over expression of reg I within AR42J did not stimulate growth. But, reg I can have an intracellular effect through a reg I-receptor-independent pathway. We recently showed that reg I directly binds to MKP-1 [21] and activate signal transduction pathways. High intracellular levels of reg I bind with MKP-1, and through subsequent modulation of the JNK and cyclin D1 pathways, regI-MKP-1 complexes can affect differentiation[21].

Normally, reg I mRNA and protein are constitutively expressed only in acinar tissue. However, during times of de-differentiation- injury, repair, and development of new endocrine and ductal cells, ectopic reg I mRNA and protein is found. Terazono et al documented that reg I is induced in beta cells of regenerating islets [22], and we noted its presence in proliferating ductules prior to the appearance of regenerating islets [3], in colonic tissue at risk for carcinoma [9], in the colons of patients with ulcerative colitis [19], and in ducts of patients with cystic fibrosis [8]. We postulate that changes in induction of reg I expression is associated with the redirection of acinar cells into endocrine and possibly ductal cells.

We have observed that cultured normal human exocrine tissue (isolated during islet cell purification) can migrate from an acinar to a ductal phenotype [8]. After one week in culture, cells expressed cytokeratin 19 mRNA and protein and decreased their expression of amylase mRNA and protein Our current data also show that lower levels of cellular reg I may be associated with differentiation into a ductal/endocrine phenotype.

Our data also indicate that higher cellular levels of reg I are associated with differentiation, or maintenance of an exocrine phenotype. This is in agreement with the established fact that reg I is constitutively expressed in acinar cells.

We previously showed [7] that administration of dexamethasone to AR42J cells decreased cellular division, increased cellular exocrine enzyme production, flattened cells to a more differentiated look with intracellular vacuoles, and decreased reg I levels as well. We did not examine cytokeratin/Pdx-1 levels in that study. While this may seem to contradict the above hypothesis, the use of dexamethasone as a stimulant may not have confounded the results, since the drug induces many changes in the cell.

Our data demonstrate that reg I over-expression is linked to the development and maintenance of the acinar cell phenotype, and depression of reg I in acinar cells can lead them to express both β-cell markers, PDX-1 and insulin. We also observed an increased cytokeratin content, which might be the result of expression of a ductal phenotype. Neuroendocrine tissues do not express cytokeratins, but acinar and ductal tissue do. The antibody panel we used contains both acinar (CK 8 and 18- mildly expressed on acinar cells) and ductal (CK19- highly expressed on ductal cells) cytokeratins, but the morphologic and biochemical movement of the cells away from the acinar state, suggests that the cells may be moving towards a ductal state. To confirm this progression to ductal tissue would require the interrogation with a CK 19 antibody [16].

So, the population of cells expressing antisense reg I may be heterogenous, with some migrating towards an endocrine phenotype and others towards a ductal one. To differentiate the subtypes, cells will be next interrogated with ductal-related genes such as CK7 and 19 and intrinsic factor [16], and endocrine-related transcription factors such as Ngn3, Pax6 and other islet hormones.

We propose that reg I expression in acinar cells is important to maintain the exocrine phenotype, as decreased expression in acinar cells can induce their dedifferentiation into ductal and/or β-cells. It is clear now that acinar cells are not terminally differentiated [11,23–26] and intracellular reg I gene expression is likely involved in the direction the cell takes.

In summary, the amphicrine cell line AR42J remains a good model to study pancreatic cell differentiation. We demonstrated that reg I over-expression is linked to a more acinar cell phenotype, and inhibition of reg I expression results in directing the cells to express beta and possibly ductal phenotypes. Acinar cells of the pancreas have been shown to transdifferentiate into beta, ductal and cancer cells, and we have shown that reg I expression in these cells is essential to maintain the exocrine phenotype.

Acknowledgments

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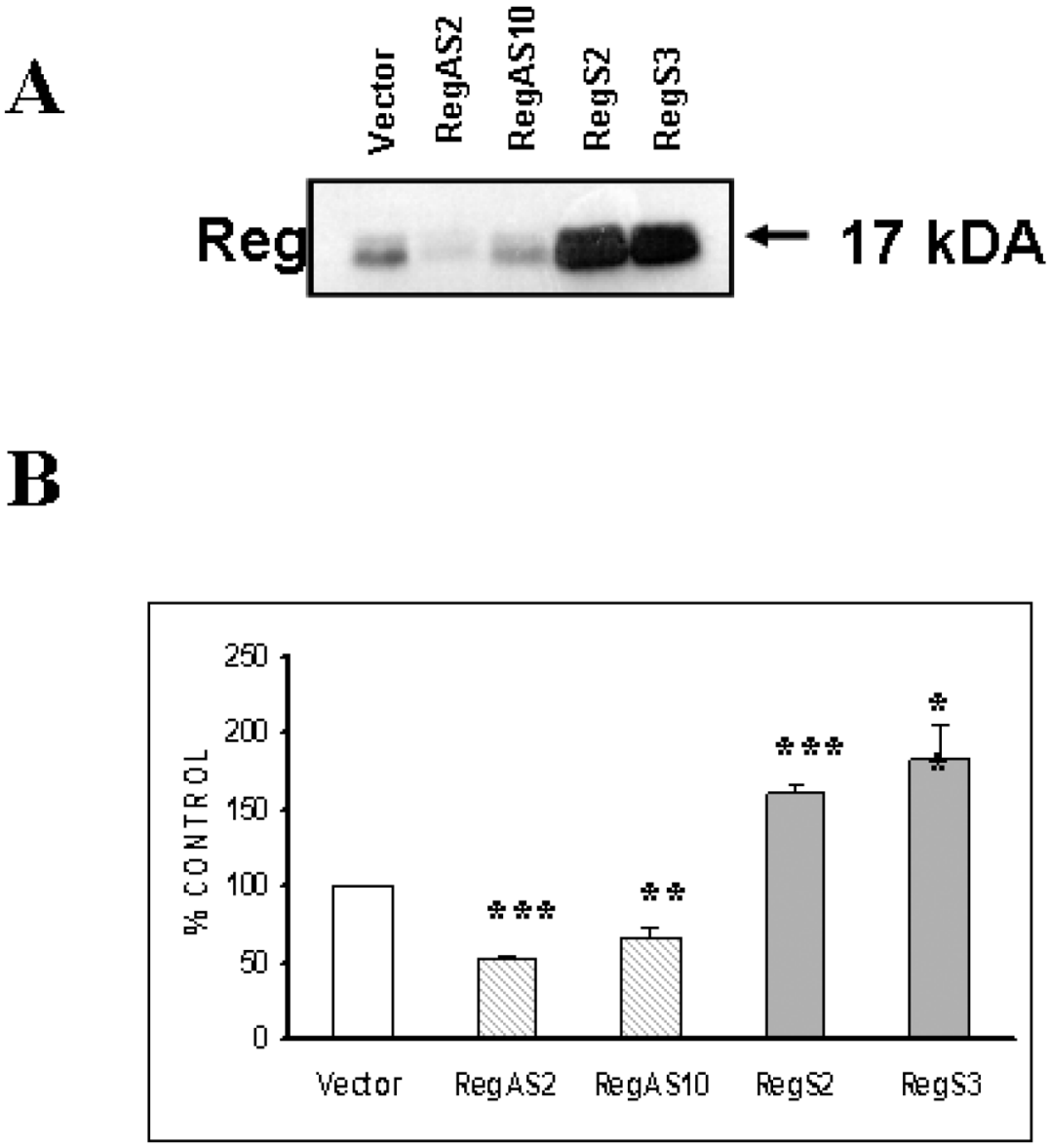

Figure 1.

Reg I protein levels of AR42J cells transfected with sense or antisense reg I pCR vector. A. Western blot analysis demonstrating changes of expression of reg I protein. Equal amounts of protein were loaded in each lane (10μg). Lane 1:AR42J transfected with empty pCR vector, Lanes 2 & 3: antisense RegAS2 and RegAS10, respectively, Lanes 4 & 5: sense RegS2 and RegS3, respectively. B. Densitometric analysis is expressed in percent change relative to AR42J transfected with empty vector. Values are expressed as mean ± SD of three separate experiments.

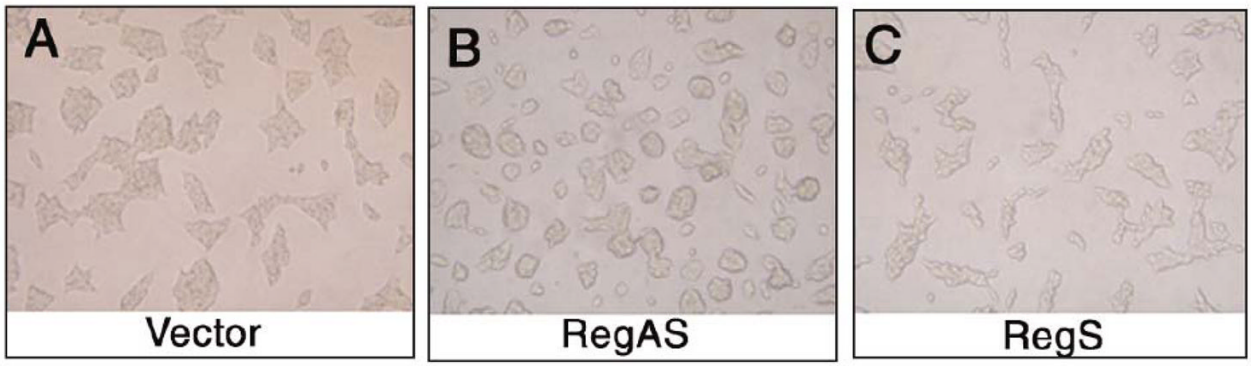

Figure 2.
AR42J morphology by phase-contrast microscopy after 5 days of culture. Equal numbers of cells were cultured under identical conditions. A. AR42J transfected with empty pCR vector, B. AR42J transfected with reg I cDNA antisense orientation. C. AR42J transfected with reg I cDNA sense orientation (×100).

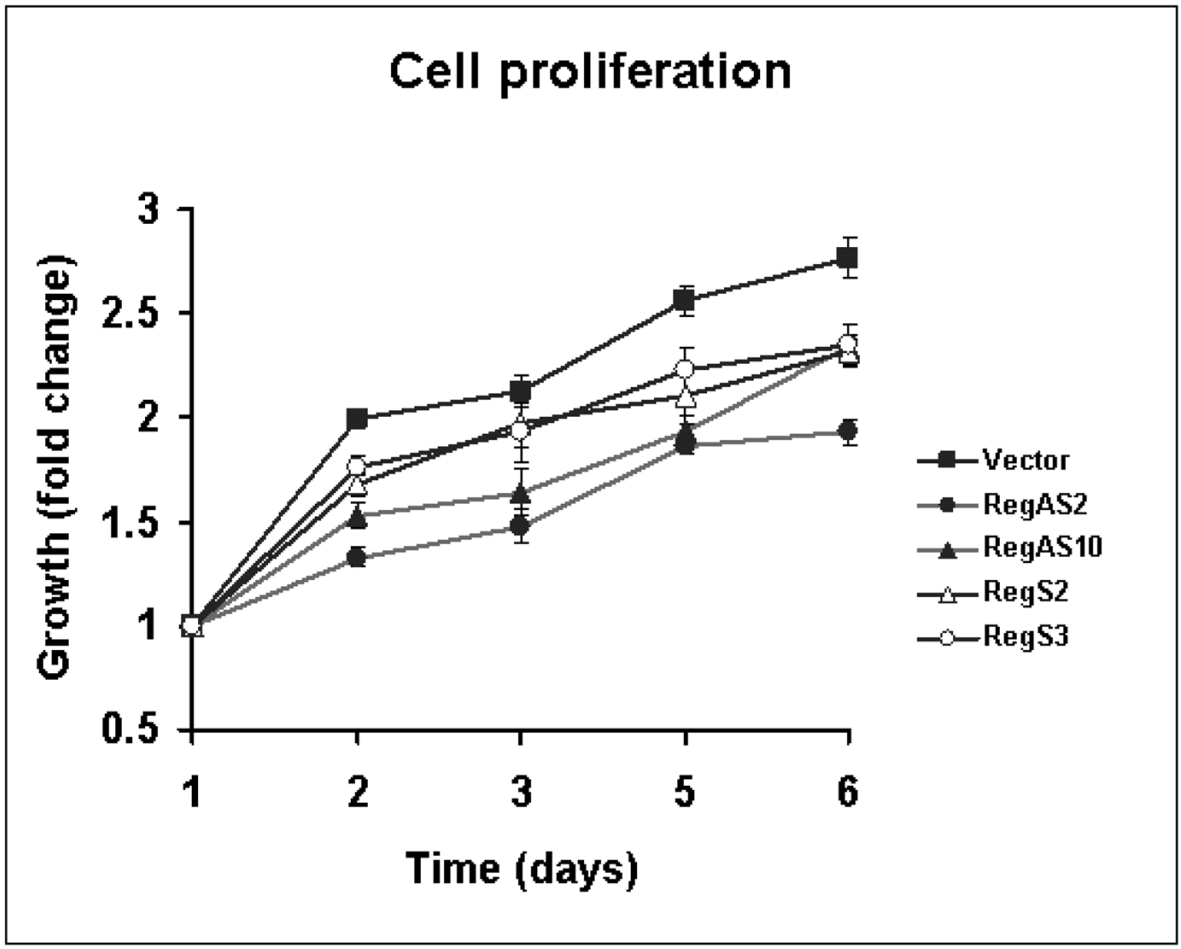

Figure 3.

Comparative growth curves of empty vector and reg I transfected cell lines. Data represent the mean ± SD fold change from day 1, 6 wells were used for each point. Every point showed significant difference from the vector on the same day of culture except on day 3 for sense RegS2 and RegS3. For example, at 6 days the fold change relative the first day, vector 2.77 ±0.10, RegAS2 1.93±0.06, RegAS10 2.33±0.04, RegS2 2.32±0.08 and RegS3 2.35±0.10 while p values were $p<0.001$, $p<0.01$, $p<0.01$ and $p<0.001$ respectively.

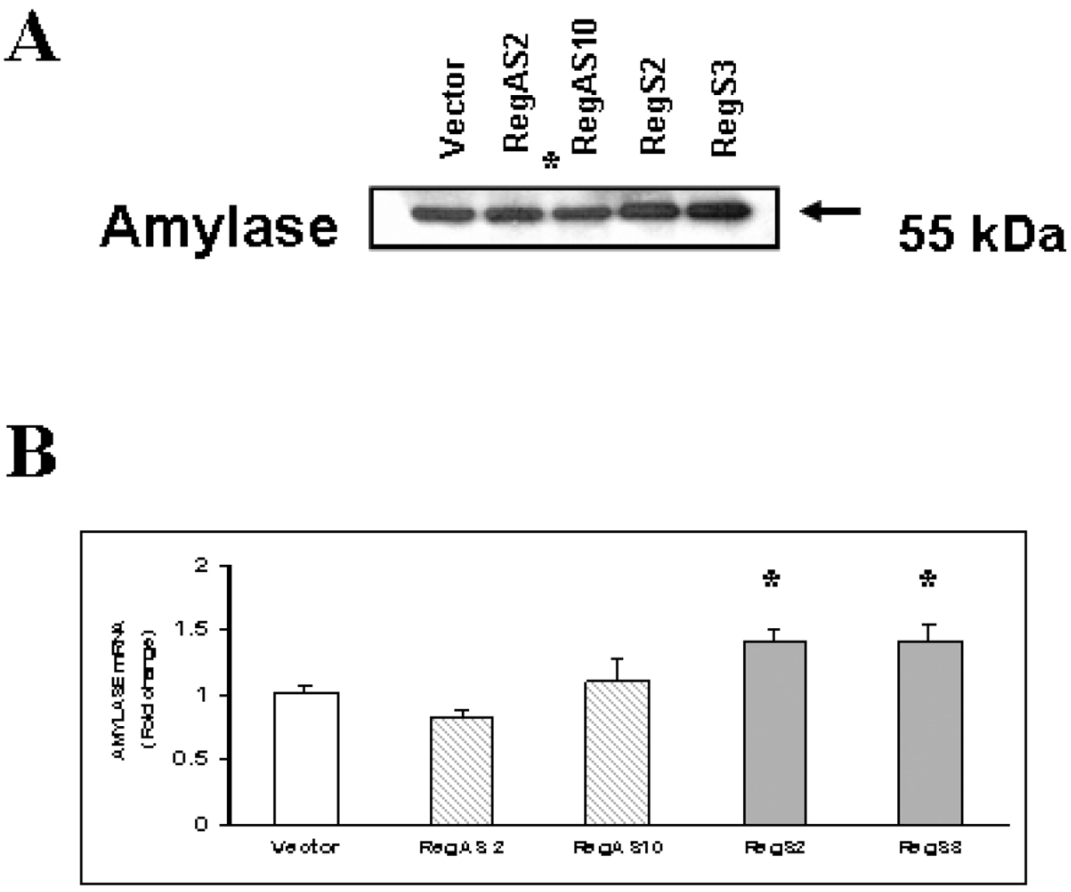

Figure 4. Level of amylase protein in transfected cell lines

A. Western blot analysis of amylase protein. Equal amounts of protein were loaded in each lane (10μg), and confirmed by b-actin blots (not shown). Lane 1: AR42J transfected with empty pCR vector, Lanes 2 & 3: antisense RegAS2 and RegAS10 clones, Lanes 4 & 5: sense RegS2 and RegS3 clones. B. Densitometric analysis is expressed in percent change relative to AR42J transfected with empty vector. Values are expressed as units of amylase per mg total protein and are mean ± SD of 5–6 independent experiments.* $p<0.01$.

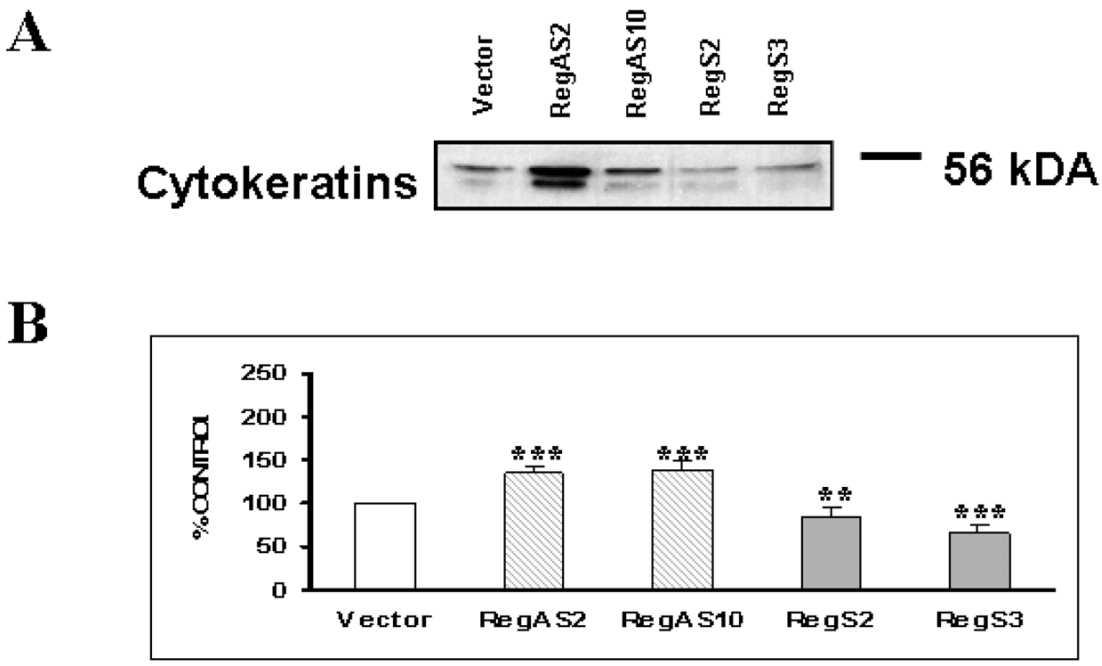

Figure 5.

Levels of cytokeratin proteins in transfected cell lines. A. Western blot analysis of protein expression of cytokeratins. Equal amounts of protein were loaded in each lane (10μg), and confirmed by b-actin blots (not shown). Lane 1:AR42J transfected with empty vector, Lanes 2 & 3: antisense RegAS2 and RegAS10, Lanes 4 & 5: sense RegS2 and RegS3. The size of cytokeratin 19 typically is 40kD. B. Densitometric analysis is expressed in percent change relative to AR42J transfected with empty vector. Values are expressed as mean ± SD of three separate experiments. ** $p<0.01$ and *** $p<0.001$.

Table 1

Real-Time quantitative Polymerase chain reaction amplification primers

Names	Sequences of primers Sense (5′) and Antisense (5′)	Sizes (bp)	Nucleotides	Gen Bank accession number
β-Actin	ACTGCCCTGGCTCCTAGCA GAGCCACCAATCCACACAGA	80	952–970 1032-1012	NM_031144
Amylase	GGACAACCATGACAATCAGCG TGGAAATTTCTTGTCCTTCGGTAAC	158	939–959 1097-1073	NM_031502
Pdx1	TTCCCGAATGGAACCGAGAC CCTCCGGTTCTGCTGCGTATG	135	398–417 533-513	NM_022852
Insulin 1&2	CCTGCCCAGGCTTTTGTCA TCCACCCAGCTCCAGTTGTG	149	61–79 210-191	NM_019130

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Gen Bank accession number: NM_019130

Table 2

Relative expression of mRNA

	Vector	RegAS2	RegAS10	RegS2	RegS3
Amylase	1.023 ±0.048	0.825 ±0.060*	1.107 ±0.171	1.410 ±0.102*	1.415 ±0.129*
Pdx-1	1.006 ±0.048	1.758 ±0.263*	2.112 ±0.203**	1.145 ±0.271	0.767 ±0.058*
Insulin	1.006 ±0.065	2.094 ±0.199*	2.422 ±0.267*	1.28 ±0.255	1.09 ±0.172

Mean ± SD,

* p< 0.05,

** p< 0.01, vs. Vector, stable transfected cell line expressing empty vector