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Assessment of Pep, Fndc5, C2c12, Pgc1 α Pattern of Genes Expression during Differentiation of Human Embryonic Stem Cells into Heart Cells

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Abstract

FNDC5 gene with another protein called Xuemei Proxy (PEP) is a 209 amino acid protein coding. These genes mainly in heart tissue, skeletal muscle and brain expressed. This study aimed to clarify the pattern of expression of this gene in mouse embryonic cells Heart cells taken. The mouse embryonic stem cells as a model for cardiac differentiation induced by ascorbic acid used and the pattern of expression of PEP at certain stages of differentiation were analyzed by Real-Time PCR technique. The results show a dramatic increase in PEP gene expression in the adult cardiomyocytes. PEP increased expression of genes may have a role in later stages cardiogenesis is possible that further studies are needed to identify it.

Keywords: Protein proxy xuemei; Cardiac differentiation; Embryonic stem cells and mice; Real-time PCR

Introduction

Fibronectin type III domain-containing protein 5, the precursor of irisin, is a protein that is encoded by the FNDC5 gene [1]. Irisin is a cleaved version of FNDC5, named after the Greek messenger goddess Iris [2] (Figures 1 and 2).

Fibronectin domain-containing protein 5 is a membrane protein comprising a short cytoplasmic domain, a transmembrane segment, and an ectodomain consisting of a ~100 kDa fibronectin type III (FNIII) domain.

FNDC5 was discovered during a genome search for fibronectin type III domains [3] and independently in a search for peroxisomal proteins [1,4].

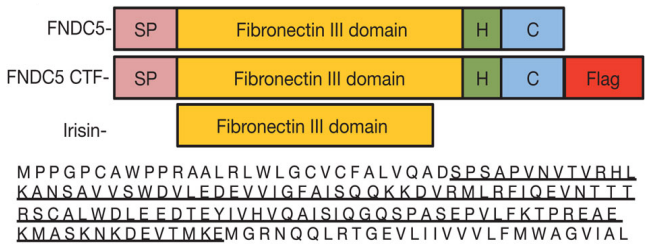

Figure 1: The amino acid sequence encoded by the gene FNDC5.

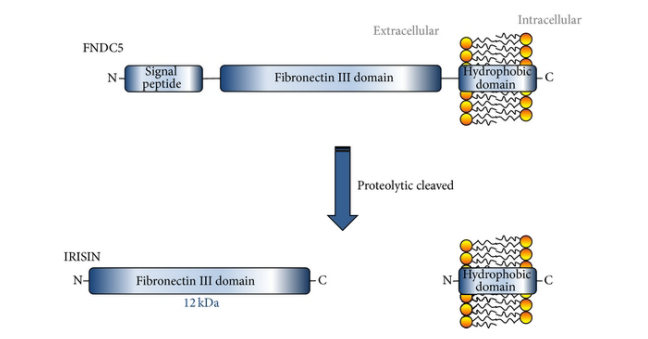

Figure 2: Structure of N-terminal and C-terminal gene FNDC5 the region.

The ectodomain was proposed to be cleaved to give a soluble peptide hormone named irisin. Separately it was proposed that irisin is secreted from muscle in response to exercise, and may mediate some beneficial effects of exercise in humans and the potential for generating weight loss and blocking diabetes has been suggested [2,5-11]. Others questioned these findings [1,12-14] (Figure 3).

The FNDC5 gene encodes a pro hormone, a single-pass type I membrane protein (human, 212 amino acids; mouse and rat, 209 amino acids) that is upregulated by muscular exercise and undergoes post-translational processing to generate irisin. The sequence of the protein includes a signal peptide, a single fibronectin type III domain and a C-terminal hydrophobic domain that is anchored in the cell membrane.

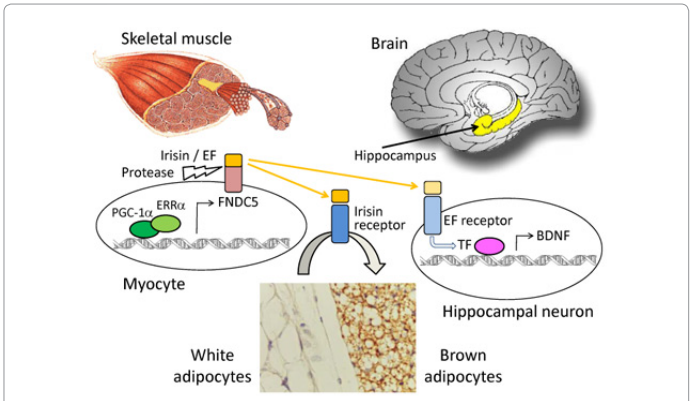

Figure 3: How FNDC5 gene and PGC1a neuron in the human brain functions.

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The production of irisin is similar to the shedding and release of other hormones and hormone-like polypeptides, such as epidermal growth factor and TGF alpha, from transmembrane precursors. After the N-terminal signal peptide is removed, the peptide is proteolytically cleaved from the C-terminal moiety, glycosylated and released as a hormone of 112 amino acids (in human, amino acids 32-143 of the full-length protein; in mouse and rat, amino acids 29-140) that comprises most of the FNIII repeat region.

The sequence of irisin, the cleaved and secreted portion of FNDC5, is highly conserved in mammals; the human and murine sequences are identical [2]. However, the start codon of human FNDC5 is mutated to ATA, which causes it to be expressed at only 1% the level of other animals with the normal ATG start - though its circulation levels are still on par with other key human hormones, such as insulin [15].

A difference in the nucleotide sequence of human FNDC5 from that of mouse Fndc5 creates a different initiation codon, potentially generating a protein that begins at methionine-76 (Met-76). A protein initiated at Met-76 would be missing the signal peptide and would be trapped in the cytoplasm. Via mass spectrometry, irisin has been found to circulate in humans in levels similar to other key hormones, such as insulin [15] (Figure 4).

Exercise causes increased expression in muscle of peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PGC-1alpha), which is involved in adaptation to exercise. In mice, this causes production of the FNDC5 protein which is cleaved to give a new product irisin [2,7]. Due to its production through a mechanism initiated by muscular contraction, irisin has been classified as a myokine [16].

Based on the findings that FNDC5 induces thermogenin expression in fat cells, overexpression of FNDC5 in the liver of mice prevents diet-induced weight gain, and *FNDC5* mRNA levels are elevated in human muscle samples after exercise, it has been proposed that irisin promotes the conversion of white fat to brown fat in humans which would make it a health promoting hormone [5,6]. However this proposal has been challenged [17] because *FNDC5* is upregulated only in highly active elderly humans [12].

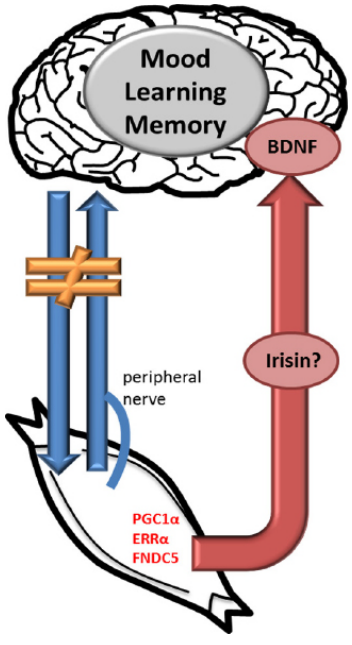

Figure 4: How FNDC5 gene and PGC1a gene function interacts with Irisin in human brain memory a cell.

A 2016 *in vitro* study of white and brown fat cell tissue found dose-related upregulation of a protein called UCP1 that contributes to the browning of white fat and found other markers that would indicate that the white cells were browning and that fat cells were more metabolically active. Many of the stem cells became a type of cell that matures into bone. The tissue treated with irisin produced about 40 percent fewer mature fat cells [18]. In mice, irisin released from skeletal muscle during exercise acts directly on bone by increasing cortical bone mineral density, bone perimeter and polar moment of inertia.

Phosphoenolpyruvate carboxykinase (PEPCK) is an enzyme in the lyase family used in the metabolic pathway of gluconeogenesis. It converts oxaloacetate into phosphoenolpyruvate and carbon dioxide [1,19,20]. It is found in two forms, cytosolic and mitochondrial.

In humans there are two isoforms of PEPCK: a cytosolic form (SwissProt P35558) and a mitochondrial isoform (SwissProt Q16822) which have 63.4% sequence identity. The cytosolic form is important in gluconeogenesis. However, there is a known transport mechanism to move PEP from the mitochondria to the cytosol, using specific membrane transport proteins.

X-ray structures of PEPCK provide insight into the structure and the mechanism of PEPCK enzymatic activity. The mitochondrial isoform of chicken liver PEPCK complexed with Mn^{2+}, Mn^{2+}-phosphoenolpyruvate (PEP), and Mn^{2+}-GDP provides information about its structure and how this enzyme catalyzes reactions [2]. Delbaere et al. resolved PEPCK in *E. coli* and found the active site sitting between a C-terminal domain and an N-terminal domain. The active site was observed to be closed upon rotation of these domains [3].

Phosphoryl groups are transferred during PEPCK action, which is likely facilitated by the eclipsed conformation of the phosphoryl groups when ATP is bound to PEPCK [3].

Since the eclipsed formation is one that is high in energy, phosphoryl group transfer has a decreased energy of activation, meaning that the groups will transfer more readily. This transfer likely happens via a mechanism similar to SN2 displacement [3].

PEPCK gene transcription occurs in many species, and the amino acid sequence of PEPCK is distinct for each species.

For example, its structure and its specificity differ in humans, *Escherichia coli* (*E. coli*) and the parasite *Trypanosoma cruzi* [4] (Figure 5).

PEPCase converts oxaloacetate into phosphoenolpyruvate and carbon dioxide. As PEPCK acts at the junction between glycolysis and the Krebs cycle, it causes decarboxylation of a C4 molecule, creating a C3 molecule. As the first committed step in gluconeogenesis, PEPCK decarboxylates and phosphorylates oxaloacetate (OAA) for its conversion to PEP, when GTP is present. As a phosphate is transferred, the reaction results in a GDP molecule. When pyruvate kinase-the enzyme that normally catalyzes the reaction that converts PEP to pyruvate-is knocked out in mutants of *Bacillus subtilis*, PEPCK participates in one of the replacement anaplerotic reactions, working in the reverse direction of its normal function, converting PEP to OAA [5]. Although this reaction is possible, the kinetics is so unfavorable that the mutants grow at a very slow pace or do not grow at all [5].

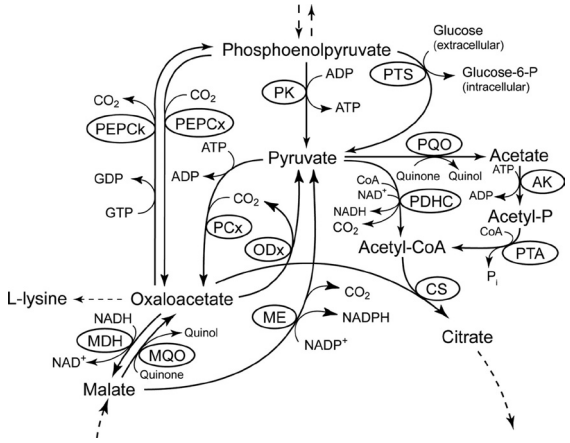

Figure 5: Phosphoenolpyruvate cycle.

PEPCK-C catalyzes the rate-controlling step of gluconeogenesis, the process whereby glucose is synthesized. The enzyme has therefore been thought to be essential in glucose homeostasis, as evidenced by laboratory mice that contracted diabetes mellitus type 2 as a result of the overexpression of PEPCK-C [6]. The role that PEPCK-C plays in gluconeogenesis may be mediated by the citric acid cycle, the activity of which was found to be directly related to PEPCK-C abundance [7].

PEPCK-C levels alone were not highly correlated with gluconeogenesis in the mouse liver, as previous studies have suggested [7]. While the mouse liver almost exclusively expresses PEPCK-C, humans equally present a mitochondrial isozyme (PEPCK-M). PEPCK-M has gluconeogenic potential per se [20]. Therefore, the role of PEPCK-C and PEPCK-M in gluconeogenesis may be more complex and involve more factors than was previously believed.

In animals, this is a rate-controlling step of gluconeogenesis, the process by which cells synthesize glucose from metabolic precursors. The blood glucose level is maintained within well-defined limits in part due to precise regulation of PEPCK gene expression. To emphasize the importance of PEPCK in glucose homeostasis, over expression of this enzyme in mice results in symptoms of type II diabetes mellitus, by far the most common form of diabetes in humans. Due to the importance of blood glucose homeostasis, a number of hormones regulate a set of genes (including PEPCK) in the liver that modulates the rate of glucose synthesis (Figure 6).

PEPCK-C is controlled by two different hormonal mechanisms. PEPCK-C activity is increased upon the secretion of both cortisol from the adrenal cortex and glucagon from the alpha cells of the pancreas. Glucagon indirectly elevates the expression of PEPCK-C by increasing the levels of cAMP (via activation of adenylyl cyclase) in the liver which consequently leads to the phosphorylation of S133 on a beta sheet in the CREB protein. CREB then binds upstream of the PEPCK-C gene at CRE (cAMP response element) and induces PEPCK-C transcription. Cortisol on the other hand, when released by the adrenal cortex, passes through the lipid membrane of liver cells (due to its hydrophobic nature it can pass directly through cell membranes) and then binds to a Glucocorticoid Receptor (GR). This receptor dimerizes and the cortisol/GR complex passes into the nucleus where it then binds to the Glucocorticoid Response Element (GRE) region in a similar manner to CREB and produces similar results (synthesis of more PEPCK-C). Together, cortisol and glucagon can have huge synergistic results; activating the PEPCK-C gene to levels that neither cortisol nor glucagon could reach on their own. PEPCK-C is most abundant in the liver, kidney, and adipose tissue [1].

A collaborative study between the U.S. Environmental Protection Agency (EPA) and the University of New Hampshire investigated the effect of DE-71, a commercial PBDE mixture, on PEPCK enzyme kinetics and determined that *in vivo* treatment of the environmental pollutant compromises liver glucose and lipid metabolism possibly by activation of the pregnane xenobiotic receptor (PXR) and may influence whole-body insulin sensitivity [8].

Researchers at Case Western Reserve University have discovered that overexpression of cytosolic PEPCK in skeletal muscle of mice causes them to be more active, more aggressive, and have longer lives than normal mice; see metabolic supermice.

PEPCK (EC 4.1.1.49) is one of three decarboxylation enzymes used in the inorganic carbon concentrating mechanisms of C4 and CAM plants. The others are NADP-malic enzyme and NAD-malic enzyme [9,10]. In C4 carbon fixation, carbon dioxide is first fixed by combination with phosphoenolpyruvate to form oxaloacetate in the mesophyll. In PEPCK-type C4 plants the oxaloacetate is then converted to aspartate, which travels to the bundle sheath. In the bundle sheath cells, aspartate is converted back to oxaloacetate. PEPCK decarboxylates the bundle sheath oxaloacetate, releasing carbon dioxide, which is then fixed by the enzyme Rubisco. For each molecule of carbon dioxide produced by PEPCK, a molecule of ATP is consumed.

PEPCK acts in plants that undergo C4 carbon fixation, where its action has been localized to the cytosol, in contrast to mammals, where it has been found that PEPCK works in mitochondria [11].

Although it is found in many different parts of plants, it has been seen only in specific cell types, including the areas of the phloem [12]. It has also been discovered that, in cucumber (*Cucumis sativus* L.), PEPCK levels are increased by multiple effects that are known to decrease the cellular pH of plants, although these effects are specific to the part of the plant [14].

PEPCK levels rose in roots and stems when the plants were watered with ammonium chloride at a low pH (but not at high pH), or with butyric acid. However, PEPCK levels did not increase in leaves under these conditions.

In leaves, 5% CO_2 content in the atmosphere leads to higher PEPCK abundance [12]. In an effort to explore the role of PEPCK, researchers

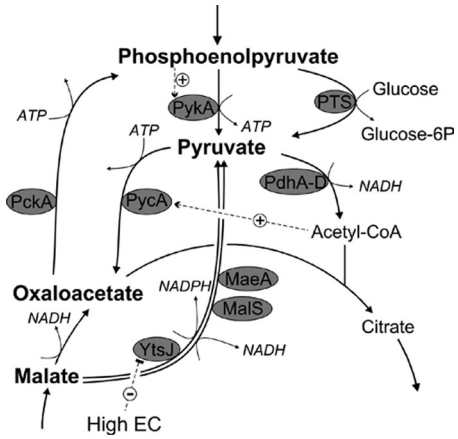

Figure 6: Phosphoenolpyruvate cycle with the release of enzymes.

caused the overexpression of PEPCK in *E. coli* bacteria via recombinant DNA [13].

PEPCK of *Mycobacterium tuberculosis* has been shown to trigger the immune system in mice by increasing cytokine activity [14]. As a result, it has been found that PEPCK may be an appropriate ingredient in the development of an effective subunit vaccination for tuberculosis [14]. PEPCK has not been considered in cancer research until recently. It has been shown that in human tumor samples and human cancer cell lines (breast, colon and lung cancer cells) PEPCK-M and not PEPCK-C, was expressed at enough levels to play a relevant metabolic role [15,19]. Therefore, PEPCK-M could have a role in cancer cells, especially under nutrient limitation or other stress conditions.

PEPCK-C is enhanced, both in terms of its production and activation, by many factors. Transcription of the PEPCK-C gene is stimulated by glucagon, glucocorticoids, retinoic acid and adenosine 3',5'-monophosphate (cAMP), while it is inhibited by insulin [16]. Of these factors, insulin, a hormone that is deficient in the case of type 1 diabetes mellitus, is considered dominant, as it inhibits the transcription of many of the stimulatory elements [16]. PEPCK activity is also inhibited by hydrazine sulfate and the inhibition therefore decreases the rate of gluconeogenesis [17].

In prolonged acidosis, PEPCK-C is upregulated in renal proximal tubule brush border cells, in order to secrete more NH_3 and thus to produce more HCO_3^- [18].

The GTP-specific activity of PEPCK is highest when Mn^{2+} and Mg^{2+} are available [13]. In addition, hyper-reactive cysteine (C307) is involved in the binding of Mn^{2+} to the active site [2] (Figure 7).

Materials and Methods

Cultured embryonic stem cells

In the study of embryonic stem cells line embryo B1 CBL576 derived from mice were used. Number 3×10^5 stem cell culture flasks on gelatinization were previously covered by feeder cells was cultured. Environment consisting of: Knockout DMEM (Gibco), 15% serum (ES-FCS, Gibco), mercaptoethanol (Sigma), 2 mM L-glutamine (Gibco), non-essential amino acids, penicillin and streptomycin, LIF (Chemicon), respectively. Embryonic stem cells are cultured with 5% carbon dioxide, 95% humidity and 37°C and kept growing.

Development and differentiation of embryoid bodies in cardiomyocytes

In an effort to evaluate the PAP gene expression pattern during differentiation into cardiac cells, mouse embryonic stem cells in culture induced by ascorbic acid adhesive was used as a model cardiogenesis. Thus, after the isolation of embryonic stem cells from fibroblasts to produce embryoid bodies hanging drop method, µL 20 droplets containing 8×10^2 cells per drop in K-DMEM Gibco, 10829-018 acid containing 15% ES-FCS ascorbic (Sigma, A4403) to a final concentration 10^{-4}M inside the bacterial culture dishes containing sterile distilled water for 2 days at 37°C and 5% carbon dioxide were cultured. Then on the seventh day 24 embryoid bodies in containers that have been coated with 1% gelatin medium without LIF and stem cells Ascorbic acid at 37°C and 5% carbon dioxide were cultured. After 5 to 7 days cardiomyocyte beating the bodies were formed (Figures 8-10).

Rate analysis

On days 12 and 14 after initiation of differentiation of embryonic bodies pulsating tests were counted using phase contrast microscopy. To assess the frequency of the pulse rate per minute was calculated. This experiment was repeated 3 times and the results were presented to the deviation from the mean. Real-Time PCR techniques for the assessment of specific gene expression PEP and cardiac markers were used. To isolate RNA from the RNase Mini Kit Qiagen kits were used. Before use, duplication, to eliminate genomic DNA contamination, RNA samples with DNase I (Fermentas) were treated for 30 min at 37°C. The EDTA samples were incubated for 5 min at 60°C to

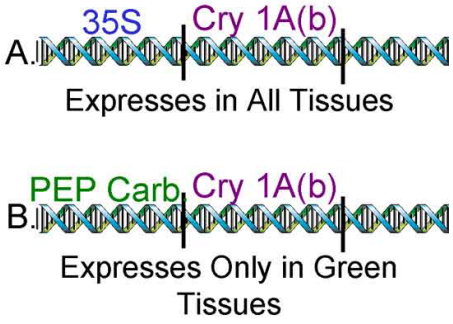

Figure 7: PEP gene expression in human tissue.

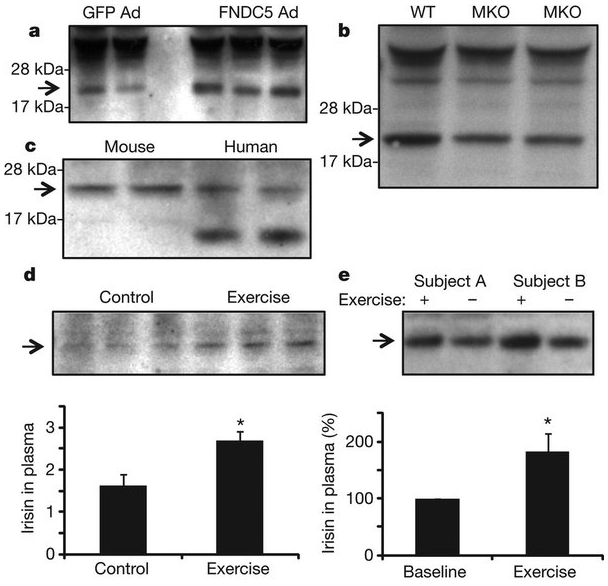

Figure 8: Pattern gang FNDC5 gene in mouse and human cells.

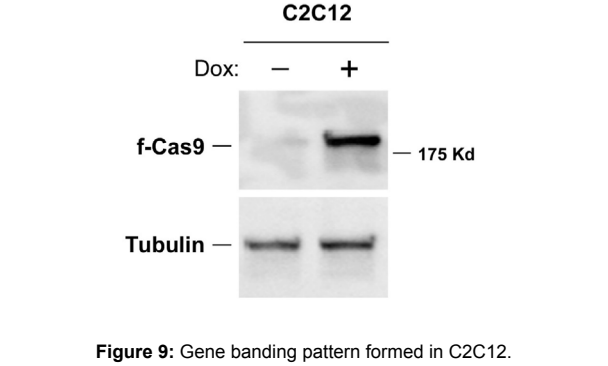

Figure 9: Gene banding pattern formed in C2C12.

inactivate the DNase. MMLV reverse transcriptase enzyme and random Hexamer and 1 mg of RNA sample were used. For Real-Time PCR reaction mixture contained 25 ng of cDNA sample and 10 ml of Rotor-Gene SYBR Green was 30 µmol of each primer pair. Expression of target genes (Table 1) Glacier aldehyde 3-phosphate dehydrogenase gene expression levels was compared.

Results

If the text was in the process of cardiac differentiation in mouse embryonic stem cells in 4 stages including mouse embryonic stem cells, embryonic bodies on the second day, the seventh day, cardiomyocytes mature EBs (pulsating bodies) were investigated. The results showed that after 7 days induced by ascorbic acid, effectively cardiomyocytes embryoid bodies differentiated mononuclear weighted with polecat have continued to beat spontaneously scattered colonies, expanded and cardiomyocytes throbbing in several areas 14 created (Table 2).

In this study, about 80% of EBs differentiated into cardiomyocytes, which was confirmed by counting the number of objects in the beating (Figures 11-18).

Discussion

Research studies show that followed the stem cells of mouse retinoic acid induced neural cells; the gene expression pattern shows an increase. It was found that the expression of cardiomyocyte clearly

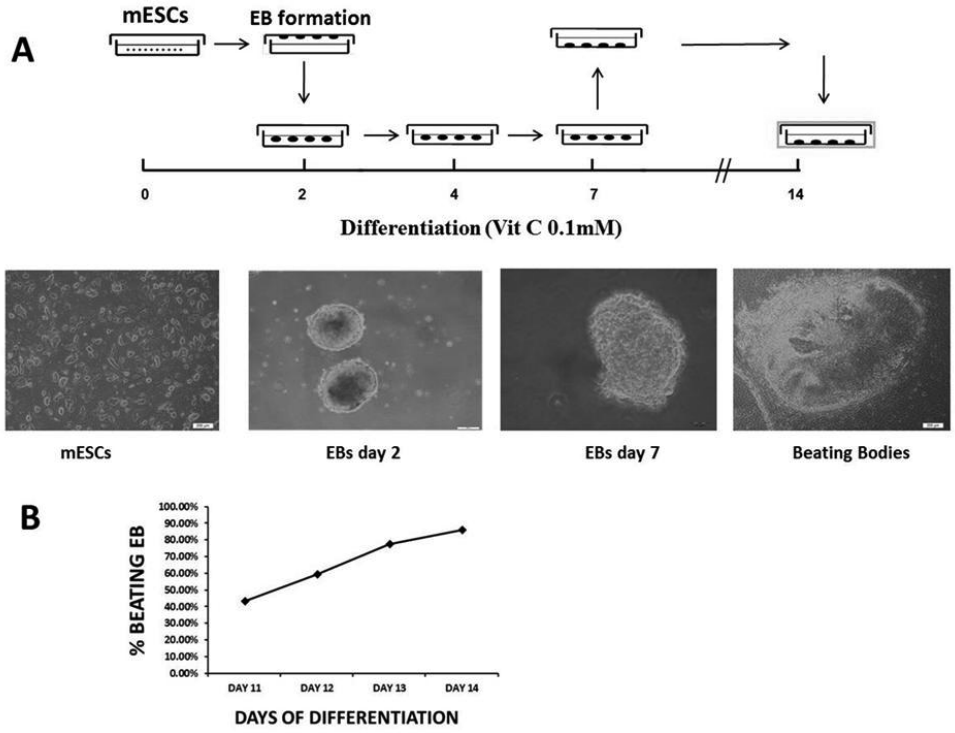

Figure 10: Stages of cardiac differentiation and cell morphology in 4 stages: stem cells, embryonic bodies, two-day, seven day old EBs and beating EBs cardiomyocytes mature and Analysis: Number of beating embryoid bodies from the eleventh to the fourteenth day is displayed.

Variety of Cell Lines	Gene Name
Stem Cells	Rex1, Nanog
Mesodermal cells	Brachyury
Cardiac precursor cells	Gata4, Nkx2.5
Adult cardiac cells	aMHC

Table 1: Marker genes for each of the cardiac differentiation of various cell lines examined and pattern gang C2C12 gene in mouse and human cells.

Gene	Forward Primer (5'-3')	Reveres Primer (5'-3')	AT	Accession No.
PEP	GACAGTAGAGAA	CCGATGATATGAG	60	NM-027402.6
GAPDH	TGCCGCCTGGAG	TGAAGTCGCAGG	60	NM-008087.4
Rex1	CCAGTCCAGAAT	AGCCATCTTCCTCA	59	NM-009557.1
Nanog	TGAGCTATAAGC	CAATGGATGCTGG	53	NM-028012.8
Brachyury	GCTCATCGGAAC	GGAGAACCAGAA	55	Nm-009408.3
Nkx5	TTAGGAGAAGGG	AGGGTGGGTGTG	4	NM-008700.4
Gata4	GGAGAAGGGCGA	TGGGTGTGAAATG	62	NM-005404.7
aMHC	CAGAGGAGAAGG	CGAACATGTGGTT	59	NM-00116417.2

Table 2: Primers used for analysis of target gene expression by real-time PCR.

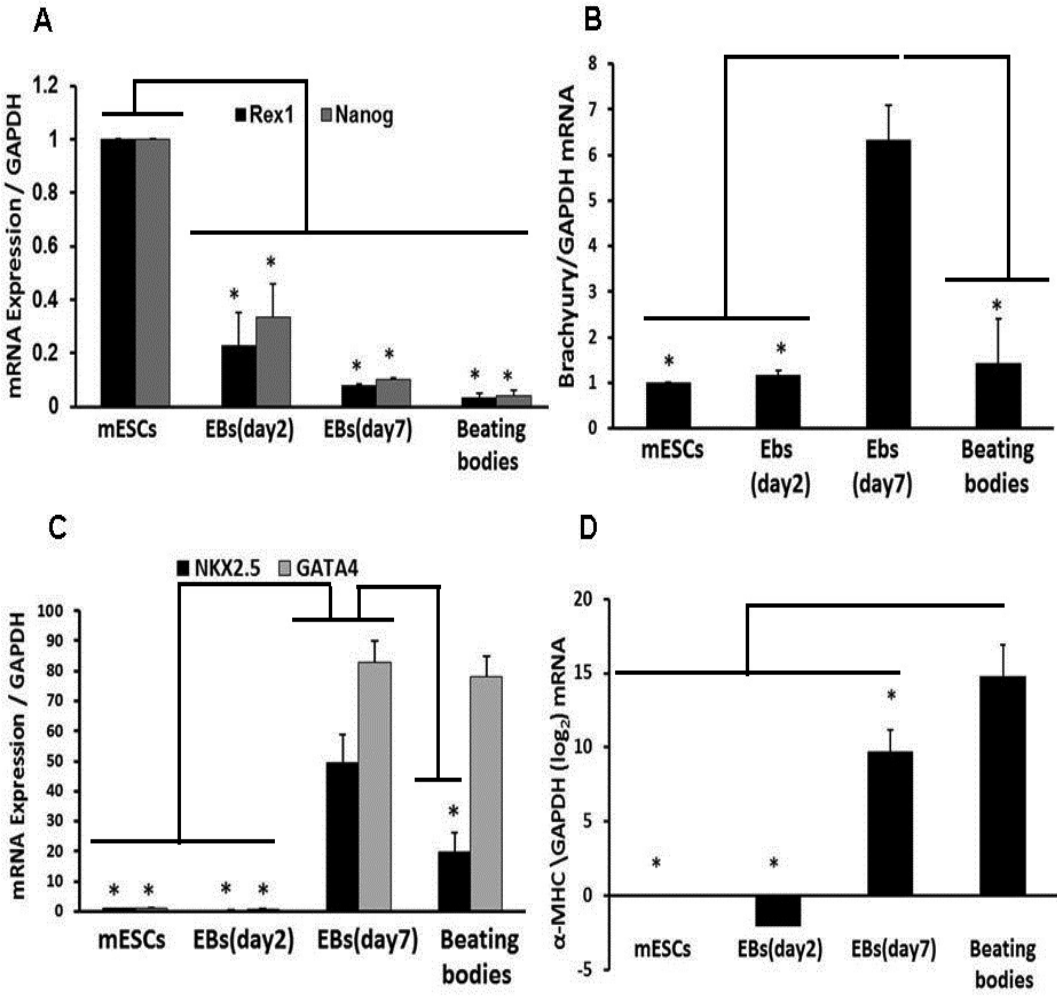

Figure 11: The pattern of gene expression during the differentiation into cardiac cells. Rex1 expression analysis and Nango (stem cell marker genes), Brachioury (mesodermal cell marker gene), Nkx2.5 and Gata4 (genetic marker for heart precursor cells) and MHC (adult cardiac cell marker gene).

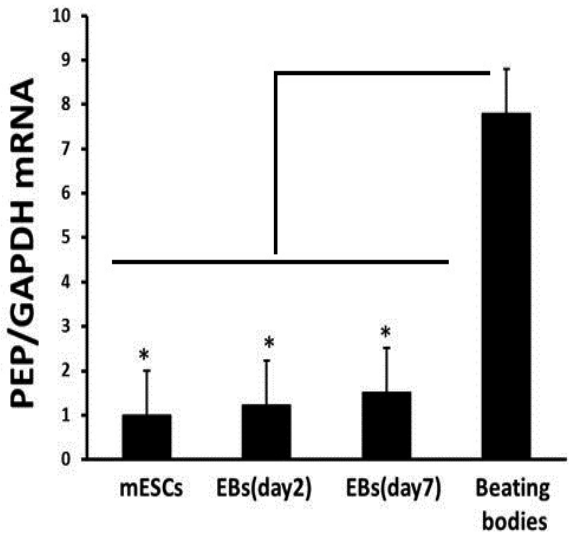

Figure 12: PEP gene expression pattern during the process of ifferentiation into cardiac cells.

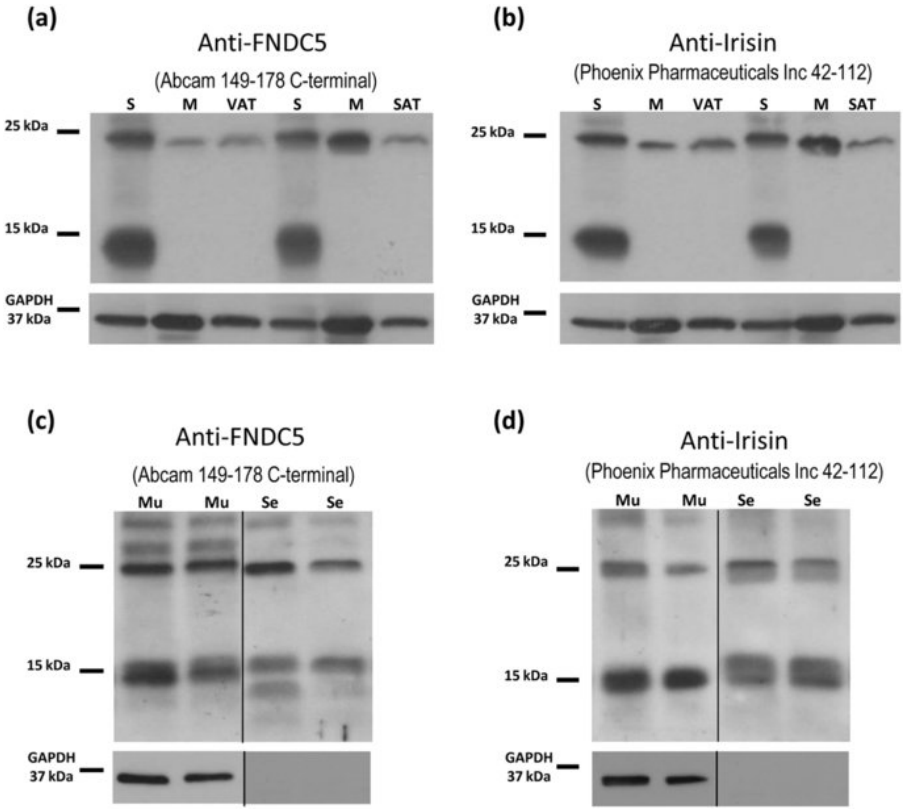

Figure 13: Pattern of gang anti-FNDC5 and anti-irisin gene in mouse and human cells.

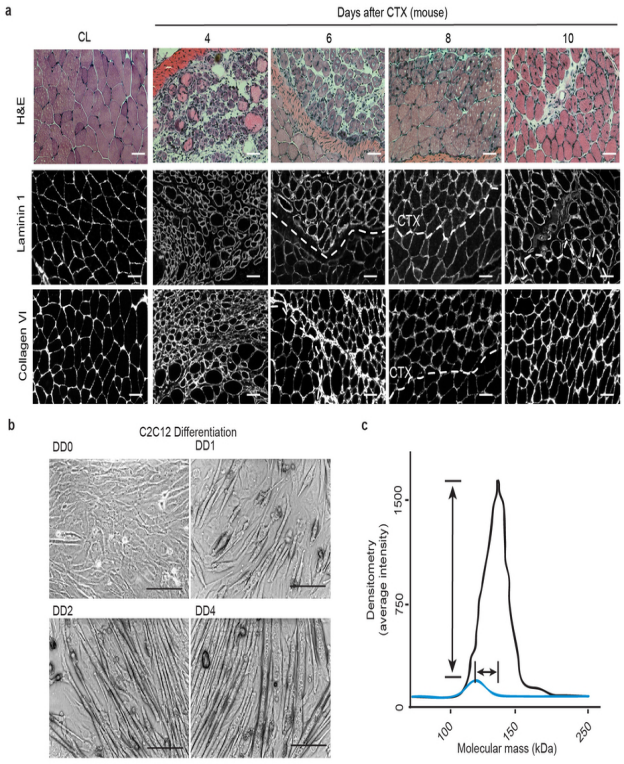

Figure 14: The pattern of gene gang C2C12 mouse cells.

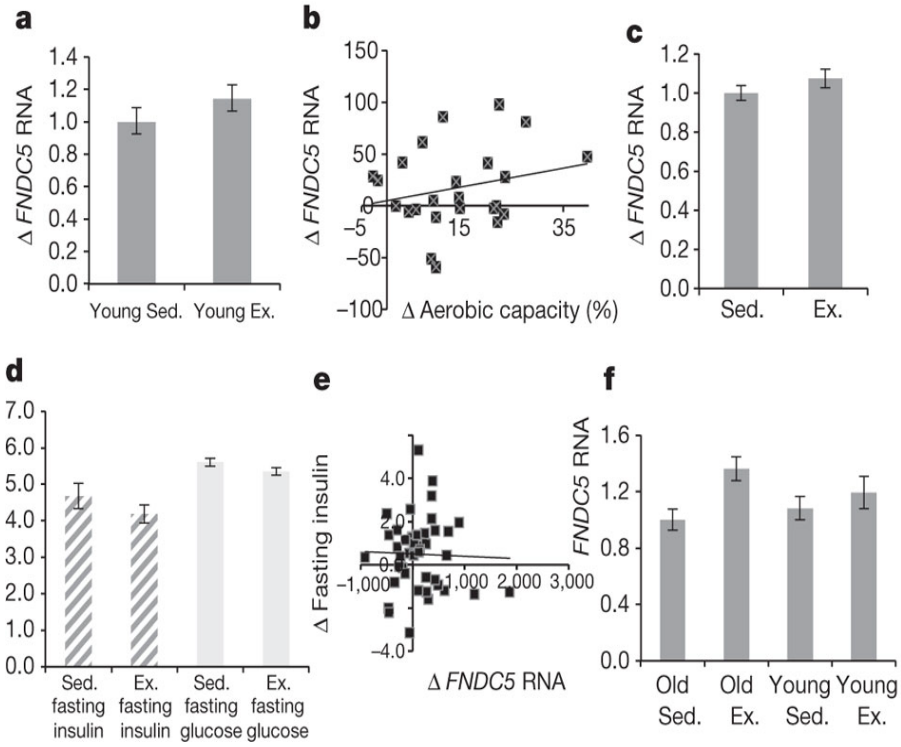

Figure 15: A comparative diagram FNDC5 RNA in cells from young and old mice.

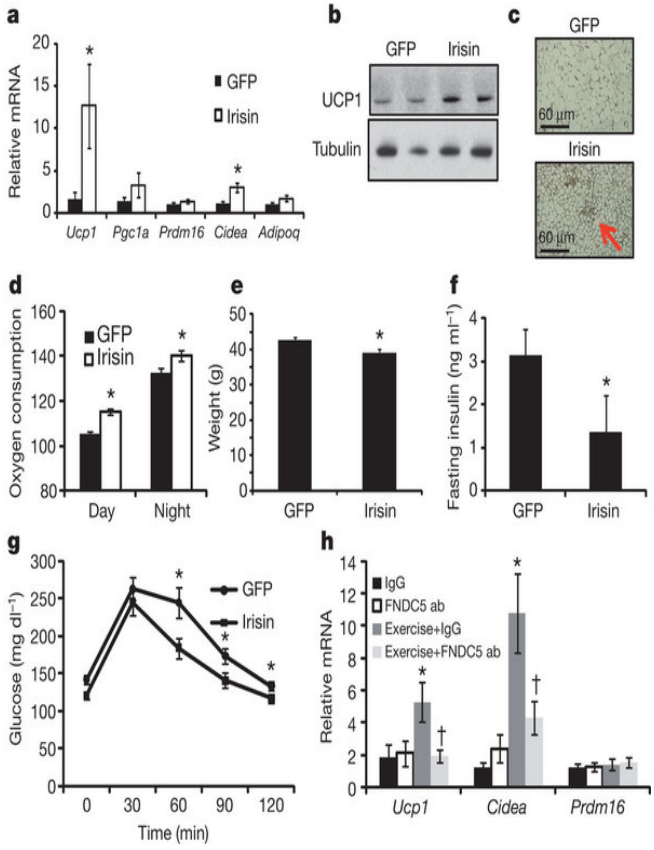

Figure 16: IRISIN FNDC5 RNA in mouse cells by oxygen uptake during the day and night.

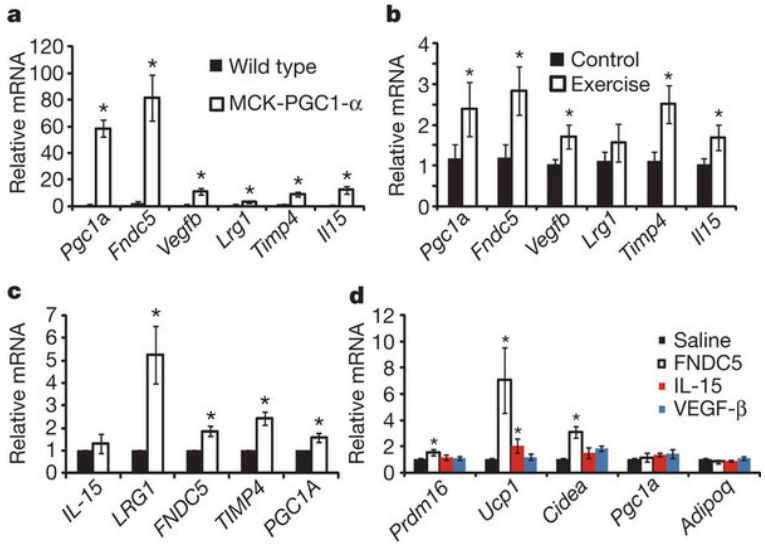

Figure 17: Diagram comparing and PGC1A FNDC5 RNA in the cells of mice created by genetic mutation and control mouse cells.

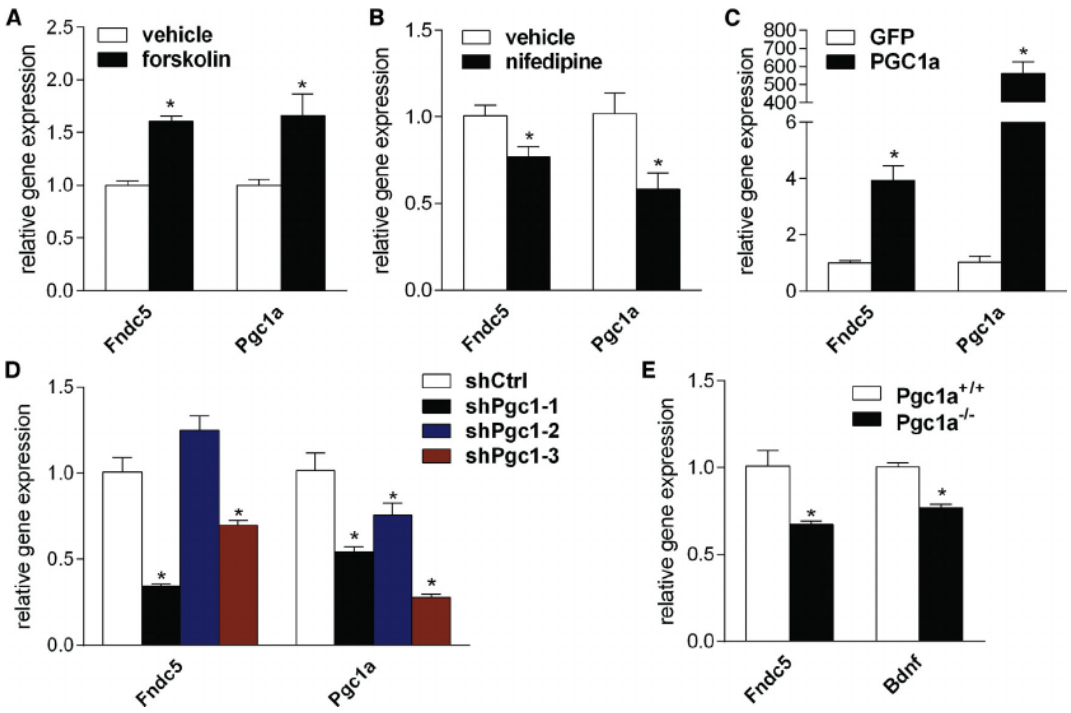

Figure 18: Diagram comparing the genes FNDC5 and PGC1A in mouse cells by the expression and activity in drug response.

increases. Due to the accumulation of EBs and the importance of ascorbic acid and ascorbic acid concentration in the study of gene expression in EBs PEP cases that require further investigation. In this study, the expression of genes in the development of embryonic heart cells analyzed the genes we would have concluded that FNDC5, C2C12, PEP and PGC1a an important role in the development of heart cells to their embryonic heart muscle.

Conclusion

It was found that the expression of cardiomyocyte clearly increases. Because of the amassing of EBs and the significance of ascorbic acid and ascorbic acid fixation in the investigation of gene expression in EBs PEP cases that require more examination, in this study, the expression of gene in the advancement of embryonic heart cells analyzed the genes we would have inferred FNDC5, C2C12, PEP, PGC1a a vital part in the improvement of heart cells to their embryonic heart muscle

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