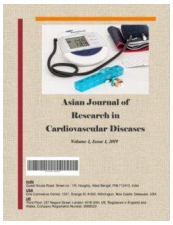

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R46K /R125K Mutation of PKP2 Instigates H9c2 Cell Hypertrophy

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Authors' contributions

This work was carried out in collaboration between both authors. Author PBA designed the project. Author DOO conducted the experiments, did the analysis and wrote the manuscripts. Both authors read and approved the final manuscript.

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Short Communication

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ABSTRACT

Plakophilin 2, an armadillo protein, is a constituent of the desmosomal protein complex. Its function is very important in the maintenance of the integrity of tissues exposed to mechanical stress, such as the cardiac tissues. Mutations in the PKP2 gene complex has been implicated in the development of (Arrhythmogenic Cardiomyopathy (ACM), a condition that predisposes athletes to sudden cardiac death, occurring mostly during sporting activities. In this study, we examined the effect of R46K-R125K mutations on the cardiac cells using H9c2 cells. Results show that H9c2 cells bearing mutant (R46K-R125K) gene for PKP2 develop cell hypertrophy after 24 hours, although the mechanisms inducing hypertrophy is currently unknown. However, R46 and R125 are known hotspots for arginine methylation, the authors propose that hypertrophy induction may be associated with factors related to defects in arginine methylation at these sites.

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Keywords: Plakophilin2; desmosomes; arrhythmogenic right ventricular cardiomyopathy; sudden cardiac death.

1. INTRODUCTION

Plakophilins are a group of proteins localized in the nuclear and desmosome complex of muscle cells. They are constituents of the link between the cytoplasmic tail of the cadherins and intermediate filament cytoskeletal proteins [1]. In order words, they function as a bridge between the junctional proteins and the cytoskeletal components of the cell. PKP2 is the gene encoding for plakophilin 2, which is a structural scaffold protein of the desmosome [2,3], and associates with nuclear and regulatory proteins to function as signalling modulator which is critical for tissue migration, differentiation and cell–cell contact formation [4]. PKP2 is also a component of the polymerase III holoenzyme, which can act as a signalling scaffold that functionally links RhoA– and protein kinase C–dependent pathways to mediate actin reorganization and regulate desmosome assembly [4]. Principally occurring in two forms (PKP2a & PKP2b), as a result of alternative splicing, PKP2 is a 100 kDa protein, sometimes appearing as a twin band on electrophoresis and made up of 837 residues [5].

Decreased expression of PKP2 in cardiomyocytes has been documented to result in decreased expression and redistribution of connexin 43, (a protein that helps in the communication of two adjacent cardiomyocytes) from the intercalated disc to the intracellular space [6]. It has been documented that dysregulation of PKP2 can cause decreased Cx43 density [7] and altered Cx43 protein expression leading to a decrease in I_{Na} amplitude, a possible cause of severe and potentially lethal cardiac arrhythmias [8]. A study by Cerrone and others in 2014 [9] demonstrated that PKP2 variants may cause sodium current (I_{Na}) deficit leading to a Brugada phenotype, thereby implying that PKP2 mutations can be a molecular substrate leading to the diagnosis of Brugada Syndrome.

There is evidence that incorporation of mutant forms of PKP2 in cardiac desmosomes can lead to disruption of cell to cell contact of adjacent cardiac myocytes and to reduced response to mechanical stress [10]. Mutations in PKP2 have also been associated with the development of (Arrhythmogenic Cardiomyopathy (ACM) [11,12, 13] a condition characterised by progressive myocardial loss and fibro-fatty replacement of cardiac myocytes, primarily affecting the right ventricle, often leading to ventricular tachycardia and sudden cardiac death, and mostly affecting athletes and young adults. The introduction of the PKP2 R735X mutation into a mice model resulted in an exercise-dependent ACM phenotype [14].

Considering the preponderance of untimely death of athletes in the field of play, potentially as a result of ACM, there is need for in depth study of mutations in the PKP2 gene, with the hope of unravelling other salient mutations that may be present in the population but hitherto unknown. The fact that R46 and R125 of PKP2 has been identified as arginine methylation sites [15], a relatively novel protein post translational modification (PTM), coupled with the fact that PTMs have been known to modulate protein function, we sort to investigate the effect of R46K_R125K mutation on H9c2 cells, by mutating arginine for lysine at 46 and 125 residues of PKP2 gene, using H9c2 cells, a cardiac-like cell, which exhibits similar hypertrophic response to neonatal cardiomyocytes as experimental models.

2. MATERIALS AND METHODS

2.1 H9c2 Cell Culture

H9c2 cells were obtained from American type culture collection (ATCC) and maintained as manufacturer's instruction. Briefly, cells were defrosted and maintained by culturing in a humidified incubator with 5% CO_2 at 37°C in a T25 cell culture flask containing Dulbecco's Modified Eagle's Medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% foetal bovine serum (Gibco, Gaithersburg, MD, USA). The media was changed after the first 24 hours, followed by 48 hrs and cells were split when 80% confluency was attained at 72 hours. Subsequent cells were split once cells attained 80% confluency. As a reserve stock, some cells were harvested, their passage number noted, and stored in a solution of 5 % dimethyl sulfoxide (DMSO) in liquid nitrogen.

2.2 Site Directed Mutagenesis

Site directed mutagenesis was carried out using the quick-change lightning site-directed mutagenesis kit (Agilent Technologies USA), oligonucleotide primers (forward & reverse) purchased from Integrated DNA Technologies (IDT), USA and the wild type templates on a pCMV6-entry vector were purchased from OriGene, UK. All protocols for polymerase chain reaction (PCR) were carried out on ice, according to manufacturer's guidelines, and the primers used are as shown below:

PKP2 R46K Forward primer = 5' GGG AGC AGC GGC AAG GGC GGC CAG ACA G 3'. Reverse primer = 5' CTG TCT GGC CGC CCT TGC CGC TGC TCC C 3'

PKP2 R125K Forward primer = 5' TGA AGG TCG CTG GGG AAA AGG AAC AGC ACA G 3'. Reverse primer = 5' CTG TGC TGT TCC TTT TCC CCA GCA ACC TTC A 3'

PCR products were digested with Dpn1 and transformed in XL10 gold ultra competent cells (Agilent Technologies USA) with Kanamycin 25ug/ml and transformed plasmids were harvested. Agarose gel electrophoresis was conducted to confirm similarity of wild type and mutant plasmids and endotoxins were removed by method of Nucleobond Xtra Midi EF (Germany) for endotoxin-free plasmid purification. The wild type and mutant plasmids were then quantified and transfected into H9c2 cells plated on cover slips in a 6-well plate. After 24 hours, cells were harvested and stained immunocytologically.

2.3 Immunocytochemical Staining

Briefly, H9c2 cells on cover slips were carefully washed three times in cold Phosphate buffered saline and fixed in 4% paraformaldehyde for 20 minutes at room temperature, followed by permeabilization in 0.1% triton-100 for 20 minutes. Cells were then blocked for 1 hour at 37 ^{0}C by incubating in a solution of 5 % ($^v/_v$) foetal bovine serum in PBS. The cover slips on which the cells were grown were carefully removed and transferred face-up unto a paraffilm film. Primary antibody (monoclonal anti-flag, F1804 Sigma-Aldrich, USA) was diluted 1:1000, and 200 µL (200 µg) was applied unto the cells and left at 4^0 C overnight. The next day, cells were washed 3 times in PBS and secondary antibody, Alexa flour 488 F(ab')$_2$ fragment of goat anti-mouse IgG (H+L), (Life technologies, USA) was applied at 1:1000 dilution and incubated in the dark for 1 hour at room temperature. Cells were washed 3 times in PBS and mounted face down onto a slide with mounting media (vector-shield) containing Dapi and the edges were sealed with nail varnish. Sections were viewed and images taken with LSM710 confocal microscope. Only H9c2 cells with green fluorescent labelling (indicating transfected cells), were analysed for cell size measurement using ImageJ.

2.4 Statistics

Results represent mean ± SEM. Comparisons were made using Student's *t*- test and results are significant when $p \leq 0.05$.

3. RESULTS

H9c2 cells transfected with the mutant and the wild type plasmids of PKP2 were stained immunocytochemically using anti-flag specific antibodies and cell size measured for a change in cell size after 48 hours. Results show that H9c2 cells transfected with mutant plasmids demonstrate increase in cell size (4140 ± 273 µm^2) compared to the wild type (3472 ± 221 µm^2) P= 0.01 (Fig. 2). PKP2 plasmids transfected into H9c2 cells resulted in the expression of plakophilin 2 proteins as green fluorescent labelling on the cell membrane.

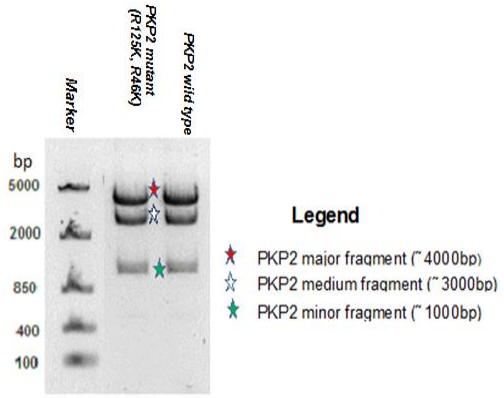

Fig. 1. DNA agarose gel electrophoreses. *Plasmids demonstrating similarity of wild type and mutant plasmids after treatment with restriction enzymes EcoR1 & Xho1.*

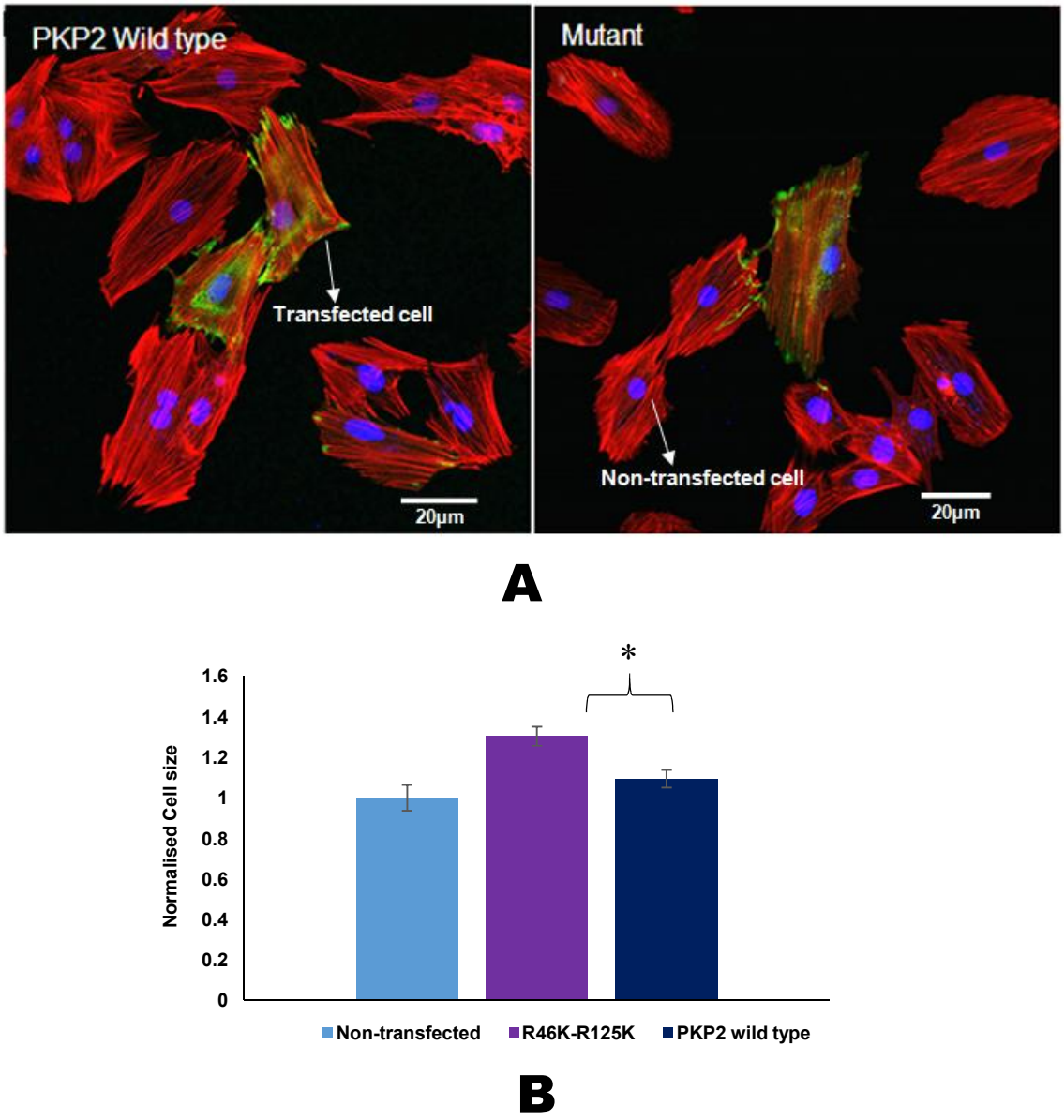

Fig. 2. Changes in cell size in wild type and mutant PKP2 plasmids

A: Confocal micrograph of H9c2 cells transfected with wild type and mutant PKP2 plasmids (Scale bar 20 µm), B: Cell size determined with Image J. Cells transfected with mutant plasmids show significant increase in cell size P=0.01, n=148 for all populations. Experiment conducted in three biological replicates

4. DISCUSSION

Plakophilin 2 is a member of armadillo family of proteins [2], and a constituent of the desmosomal protein complex, which is critical to the integrity of tissues exposed to mechanical stress. PKP2 occurs at the intercalated disc of cardiac cells and is necessary for normal morphogenesis and function of the heart [16]. Results for PKP2 proteins over expressed in H9c2 cells reveal a cytoplasmic localisation, similar to the expression in human cell line A-431 as documented by human protein atlas (http://www.proteinatlas.org/ENSG00000057294-PKP2/cell#img). This result agrees with the work of [16], who documented PKP2 localisation in the nucleus, cell membrane as well as in the cytosol of most cells. Rickelt [17] also reported similar

expression of PKP2 in MCF-7 breast cancer cell lines. In the developed heart, plakophilin 2 proteins are located at the longitudinal ends of the myocyte [18], hence the widespread cytosolic localization of PKP2 in H9c2 cells may be because of the immature nature of H9c2 cells. This is supported by the finding that at immature stages, intercalated disc proteins are not arranged at the longitudinal ends of the cell, but are rather spread across the entire cytosol [19].

PKP2 mutations accounts for 30-40% of all ARVC cases [20]. Mutations at arginine residues in PKP2 have not been reported as a cause of hypertrophic cardiomyopathy, we therefore report here for the first time, that mutations R46K-R125K in PKP2 lead to hypertrophy of cardiac-like cells. It is however unknown if the increase in cell size recorded in this work was because of lack of arginine methylation or a change in the protein structure, resulting from replacing arginine (a bulky amino acid) with lysine. Although both arginine (R) and lysine (K) belong to the same group of basic amino acids, as they are both positively charged, with basic side chains. They are often present in areas exposed to protein surfaces, binding or active sites. They can participate in forming electrostatic interactions with other residues, thereby helping in stabilising protein structure. For example, arginine and lysine play a very important role in protein structure as they are frequently involved in forming salt bridges where they pair with negatively charged amino acids such as aspartate. Arginine however possess a guanidinium group which allows interactions in three possible directions, thereby forming a larger number of electrostatic interactions when compared to lysine. It is conceivable that a replacement of arginine with lysine can introduce structural distortions in the enzyme recognition site.

Furthermore, arginine methylation, catalysed by PRMTs has been known to increase the mass, steric hindrance and hydrophobicity of the target protein, and subsequently modulate protein function. Although lysine is often methylated, there are different Tudor domain proteins that recognise and interact only with methylated arginine and not methylated lysine [21,22], hence lysine methylation cannot substitute for arginine methylation. Each Tudor domain is specific for one type of methylation (arginine or lysine) and currently there is no known example of one domain recognizing both arginine and lysine methylations [18]. It follows that an arginine – lysine mutation can preclude a protein-protein interaction as a result of Tudor proteins for methyl arginine not recognising and interacting with methyl lysine.

5. CONCLUSION

This work have shown for the first time, that an R46K_ R125K mutation in PKP2 leads to hypertrophic response in H9c2 cells, although, the mechanism for the observed hypertrophy is currently unknown. The authors propose that the hypertrophy of H9c2 cells recorded in this work can occur in animal model; hence a further study is warranted to unravel the mechanism for the induction of hypertrophy in H9c2 cells transfected with mutant R46K-R125K PKP2 plasmids.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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