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Original Research

Differential Gene Expression Signatures and Cellular Signaling Pathways induced by Lamin A/C Transcript Variants in MCF7 Cell Line

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Abstract

Background: Lamins are the major component of nuclear lamina. Alternative splicing of the 12 exons comprising *lamin A/C* gene creates five known transcript variants, lamin A, lamin C, lamin AΔ10, lamin AΔ50, and lamin C2. The main objective for this study was to examine the association of critical pathways, networks, molecular and cellular functions regulated by each Lamin A/C transcript variants. **Methods**: Ion AmpliSeq Transcriptome Human Gene Expression analysis was performed on MCF7 cells stably transfected with lamin A/C transcript variants. **Results**: Lamin A or lamin AΔ50 upregulation was associated with activation of cell death and inactivation of carcinogenesis while both lamin C or lamin AΔ10 upregulation activated carcinogenesis and cell death. **Conclusions**: Data suggest anti-apoptotic and anti-senescence effects of lamin C and lamin AΔ10 as several functions, including apoptosis and necrosis functions are inactivated following lamin C or lamin AΔ10 upregulation. However, lamin AΔ10 upregulation is associated with a more carcinogenic and aggressive tumor phenotype. Lamin A or lamin AΔ50 upregulation is associated with a predicted activation of increased cell death and inactivation of carcinogenesis. Thus, different signaling pathways, networks, molecular and cellular functions are activated/inactivated by lamin A/C transcript variants resulting in a large number of laminopathies.

Keywords: lamin A/C transcript variants; senescence; laminopathies; nuclear lamins; Ion Torrent

1. Introduction

Lamins are architectural protein components of the cell nucleus. Because of their ability to polymerize, they form molecular networks that anchor nuclear embedded proteins and peripheral chromatin components within the nuclear envelope, which confers mechanical stability, and thus have been implicated in the role of maintaining the structural integrity and overall mechanical stability of the nucleus [1]. Lamins participate in diverse nuclear cell functions including maintenance of the genome in a specific structural organization [2–5]. Lamins have also been shown to play a pivotal role in DNA repair, replication, and transcription, and thus affect cellular differentiation, apoptosis, and cell aging. Furthermore, lamins are classified as type V intermediate filament proteins, which can be categorized according to their sequence and structural organization as either A-type or B-type lamins [5]. The A-type lamins have 2 major isoforms (lamin A and lamin C) and three minor isoforms (lamin AΔ10, lamin AΔ50 [Progerin] and lamin C2), while the B-type lamins have two major isoforms (lamins B1 and B2). A-type lamins are expressed mostly in somatic cells, whereas B-type lamins are usually ubiquitously expressed and interact in the nuclear lamina's assembly process [5].

Lamin A, Lamin C, along with Progerin (Lamin AΔ50), Lamin AΔ10, and Lamin C2 (specific to the testis) are all derived from a single gene (*Lamin A/C*) by alternative splicing of one transcript of the gene, which contains exons 1 through 12 [2,3,6]. Consideration of Lamins as individual disease causing elements within the cell and nuclear lamina requires a deeper understanding of the Lamina network. The first 566 amino acids of human Lamin A/C, spanning exons 1–10, are identical in lamin A and lamin C. However, lamin C has six unique carboxyl-terminal amino acids [7]. Both Lamins A and C have been given the role of establishing nuclear mechano-transduction and stiffness, however, it has been found that Lamin C correlates more with mechanical properties than Lamin A. Unlike Lamin A, which has a prelamin that undergoes tail domain cleavage modification once it is inside the nucleus, Lamin C is the only Lamin that does not undergo post-transitional modifications with a farnesylated tail domain. Consequently, its expression is unaffected by certain mutations that occur in genes affecting farnesylation, which is a feature of Lamin A-specific disease [8].

Lamin A/C mutations has been associated with more than 300 diseases that vary in phenotype and penetrance, called laminopathies. Depending on the mutation involved, laminopathies affect different tissues in the body such as muscles, cardiomyocytes, adipocytes, and neurons. They have also been associated with known diseases such as Emery-Dreifuss muscular dystrophy and Hutchinson-Gilford progeria syndrome (HGPS) [9]. HGPS results from a single nucleotide substitution in exon 11 of *Lamin A/C* gene resulting in the usage of an alternative splice donor site in exon 11, with subsequent Progerin production which is missing 150 nucleotides (50AA). The most prevalent mutation is a change of bases C and T in exon 11, which leads to a splicing defect, thus activating the exonic cryptic donor splice site [10]. The expression of Progerin weakens the nuclear lamina, which results in misshapen nuclei, and adversely altering mitosis and cell proliferation. The accumulated Progerin eventually causes nuclear blebbing, DNA damage, and rapid telomere shortening with subsequent p53-dependent premature senescence [10,11]. Therefore, HGPS patients, who have abnormal Progerin levels, suffer premature aging and death nearly at age of 13 due to heart attack or stroke [11]. Aside from the laminopathies mentioned above, there has been an on-going investigation of the relationship between changes of the expression of some lamins that result in diverse types of cancer. Cancer cells are often characterized as highly proliferative with unregulated signaling. Thus, lamins are thought to be responsible for the structural alteration in cells undergoing malignant transformation, since they are responsible for partial cell functions regulation [2,9,12]. In tumor cells, improper expression of lamins and its interaction with other proteins are evident [12–14]. Lamin AΔ10 is another product of *Lamin A/C* alternative splicing, missing exon 10 (90 nucleotides). Upregulation of lamin AΔ10 which is found in relatively high amounts in lung cancer cell lines, resulted in distorted nuclear phenotypes [6]. MCF7 is a "Luminal A" subtype of noninvasive breast cancer derived cell line and was chosen in this *in vitro* study because MCF7 cells maintain characteristics similar to mammary epithelium. In addition, MCF7 has been utilized in several *in vitro* studies for the development of chemotherapeutic drugs and understanding drug resistance as it is highly responsive to chemotherapy [15].

The lack of consensus on *lamin A/C* effects in cancer is caused by several factors including heterogenous expression of *lamin A/C* transcript variants in tumors which may play various roles in cancer development and progression [12] and thus resulting in the activation/inhibition of various cellular signaling pathways. This study, investigated the critical cellular signaling pathways in breast cancer MCF7 cell line transfected with one of the four *lamin A/C* transcript variants (Lamin A, Lamin C, Lamin AΔ10, and Lamin AΔ50) and their possible contribution to the heterogeneity and metastatic aspects of breast cancer.

2. Materials and Methods

2.1 MCF7 Cell Line Maintenance and Transfection

Authenticated MCF7 human breast cancer cell line was purchased from ATCC (ATCC® HTB-22), American Type Culture Collection, Manassas, VA, USA) and cultured in Eagle Minimum Essential Medium (MEM) supplemented with various components, including L-glutamine (2 mM), sodium bicarbonate (1.5 g/L), 1X Non-Essential Amino Acids (NEAA), 1X Penicillin/Streptomycin (Invitrogen Inc., Carlsbad, CA, USA), sodium pyruvate (1.0 mM), and 10% fetal bovine serum (FBS) (Invitrogen Inc., Carlsbad, CA, USA). MCF7/ADR was a generous gift from Dr Abdelhadi Rebaa (Department of Pediatrics, Children's Memorial Research Center, Northwestern University). MCF-7/ADR cells have been widely used as a multidrug-resistant breast cancer cell. However, the real origin of MCF-7/ADR cells remains unclear and the MCF-7/ADR were re-designated as NCI/ADR-RES as DNA fingerprinting analysis showed NCI/ADR-RES to be unrelated to MCF-7 and NCI/ADR-RES are found to be derived from OVCAR-8 human ovarian carcinoma cells [16,17].

All cells were grown in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. For transfection, MCF7 cells were transfected with different GFP-tagged lamin A/C transcript variants using Lipofectamine™ 2000 Transfection Reagent (Cat. No. 11668027, ThermoFisher Scientific, Waltham, MA, USA). The GFP-tagged lamin AΔ50 was obtained from Addgene.org (Plasmid #17653), while the C-terminal GFP-tagged lamin A, lamin C, and lamin AΔ10 were purchased from OriGene (Rockville, MD, USA). The transfection efficiency of MCF7 cells was determined to be 80%, and stably transfected MCF7 cells were selected with G418 antibiotic (Cat. No. A1720-100MG, Invitrogen Inc.) for 14 days to establish stable cell lines expressing the transfected genes. To inhibit lamin A/C transcript variants, pSilencer 4.1-CMV neo vector (Ambion, Inc., Austin, TX) was used for siRNA-mediated knockdown. The vector contained hairpin siRNA templates targeting exon 1 (5′-GCAAAGTGCGTGAGGAGTT-3′) or exon 10 (5′-ATGAGGATGGAGATGACCT-3′) of lamin A/C to inhibit the expression of all lamin A/C transcript variants or all except lamin AΔ10, respectively. A control vector expressing a hairpin siRNA with limited homology to any known human sequences was also used as a control in the experiments. Cells were routinely tested for mycoplasma by PCR and G418 sulfate is toxic to bacteria and yeast.

2.2 mRNA Quantification of Lamin A/C Transcript Variants by RT-qPCR

Total RNA was isolated from the samples using the Ambion Aqueous kit. The quantity and quality of the isolated RNA were assessed using the Bio-Rad Experion automated electrophoresis system (Hercules, CA, USA). For cDNA synthesis, 1 µg of total RNA was reverse transcribed using SuperScript III First-Strand Synthesis kit

(Clontech Mountain View, CA, USA). PCR amplification was performed using Cepheid Smart Cycler (Sunnyvale, CA, USA). PCR mixture was prepared and run according to previously described method [14]. The amplification of lamin A/C transcript variants was performed using specific primer sequences listed in Table 1. The PCR protocol consisted of an initial activation cycle at 95 °C for 2 minutes, followed by 40 cycles of denaturation at 95 °C for 15 seconds, and annealing/extension at specific temperatures for each transcript variant (lamin A: 58 °C, lamin AΔ50: 60 °C, lamin AΔ10: 66 °C, lamin C: 60 °C) for 2 minutes. All PCR results were normalized to the expression of Cyclophilin A (CypA).

2.3 Fluorescence Confocal Microscopy

Transfected MCF7 cells were grown on plastic cover slips (18 $\times$ 22 mm) until they are 60–70% confluent. The cells were formalin (3.7%) fixed for 10 minutes at room temperature. Following fixation, the cover slips were then washed three times with PBS. Leica SP5SM confocal microscope was used for the morphometric evaluation of the nuclear localization distribution of lamin transcript variants.

2.4 Cell Proliferation Assay

Stably transfected MCF7/ADR cells with lamins siRNA targeting different exons of lamin A (exon 1 or exon 10) were incubated with Doxorubicin (Cat. NO. 324380, MilliporeSigma, Darmstadt, Germany) for 48 hours in 96-well plate. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) cell proliferation assay (abcam, Shanghai, China) was used to quantitatively estimate percentage of viable cells according to the manufacturer's protocol.

2.5 Senescence Analysis

Cellular senescence of stably transfected MCF7/ADR cells with lamins siRNA targeting different exons of lamin A (exon 1 or exon 10) was compared by histochemical staining of Senescence-associated beta-galactosidase (SA-β-gal) activity. Cultured cells were trypsinized, counted, and plated at the same cell numbers and incubated for 48 hours at 37 °C in the presence and absence of doxorubicin at different concentrations. Cells were gently rinsed once with phosphate buffered saline (PBS, pH 7.4) and then fixed (0.2% gluteraldehyde and 2% formaldehyde) at room temperature for 15 minutes followed by three washes in PBS. β-Gal Staining kit (Roche, Indianapolis, IN, USA) was used for cellular senescence determination according to the manufacturer's instructions. Cells were viewed using phase-contrast microscope (Axio Vert.A1 Zeiss, Jena, Germany). Images of SA-β-gal positive staining areas were evaluated by ImageJ (version 1.53) software (Bethesda, MD, USA. Department of Health and Human Services, National Institutes of Health).

2.6 Ion Torrent Analysis

2.6.1 RNA Extraction and Quality Control

Nanodrop ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was utilized initially to examine the quantity and the quality of the extracted total RNA as described in section 2.2. Samples with high purity (260 nm/280 nm $\geq$2) were selected for RNA-Seq. RNA Nano 6000 chips and Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) were used to check the integrity and quantity of RNA (RNA samples with RNA Integrity Number (RIN) $\geq$8 were used). Poly(A) messenger RNA was isolated using RiboMinusTM Eukaryote System v2 (Thermo Fisher Scientific, Inc.) followed by RiboMinus™ Magnetic Bead Cleanup Module (Thermo Fisher Scientific, Inc.). Qubit™ RNA HS Assay kit (Cat. No. Q32855, Thermo Fisher Scientific, Inc.) was used to quantify the isolated mRNA.

2.6.2 Library Construction for Next-Generation Sequencing (NGS)

NGS library was constructed using Ion Total RNA-Seq Kit v2 (Cat. No. A27752, Thermo Fisher Scientific, Inc.), Ion Total RNA-Seq Primer Set v2 (Cat. No. A27896, Thermo Fisher Scientific, Inc.), and Ion Express™ RNA-Seq Barcode 01-16 Kit (Cat. No. 4471252, Thermo Fisher Scientific, Inc.). The qualified library was sequenced by injecting onto the Ion PI™ Hi-Q™ Chip Kit v3 (Cat. No. A26769, Thermo Fisher Scientific, Inc.) and insertion into the Ion Chef™ Instrument (Thermo Fisher Scientific, Inc.) to emulsify and enrich the library. Samples were sequenced by the use of Ion Proton Semiconductor Sequencer (Thermo Fisher Scientific, Inc.).

2.6.3 Data Analysis

Data extraction was performed using Agilent Feature Extraction Software, version 11.0.1.1 (Agilent Technologies, Inc. Santa Clara, CA, USA), from the raw data. The extracted data were then loaded into the GeneSpring GX software, version 12.1 (Agilent Technologies, Inc.), both products of Agilent Technologies, Inc. The loaded data were log2 transformed and normalized using the percentile shift algorithm, with the percentile target set at 75. Differentially expressed genes were identified by comparing the gene expression levels in MCF7 cells transfected with one of the lamin A/C transcript variants to those in mock transfected MCF7 cells. Genes with a fold change (FC) of 2.5 or above, and with a p-value $\leq$ 0.05 were considered as differentially expressed genes. This approach allowed for the identification of genes that showed significant changes in expression levels following upregulation of lamin A/C transcript variants compared to mock transfected cells, with a focus on genes with a fold change of 2.5 or higher and statistically significant p-values. SigmaStat software (Version 3., Systat Software Inc., Westminster, CA, USA) was utilized to perform a comparison of expression levels using an

unpaired *t*-test with statistical significance set at p-value $\leq$ 0.05.

2.6.4 Ingenuity Pathway Analysis (IPA)

The association of critical pathways, networks, molecular and cellular functions with differentially expressed genes was analyzed using IPA Core analysis suite (Qiagen Biosciences Inc., Hamburg, Germany). IPA core analysis with a cut-off –log(p-value) >3 was used and the significance of IPA core analysis was measured by a Fischer's exact test or Z-score to provide predictions (activation/inactivation/no effect).

3. Results

3.1 Upregulation of Lamin A/C Transcript Variants following MCF7 Transfection

Expression analysis of total lamin A/C transcript variants was detected by measuring forward and reverse primers located in exons 1 and 7 respectively following MCF7 transfection with C-terminal GFP-tagged lamin A (Fig. 1A). Specific primers for lamin C, lamin AΔ10 and lamin AΔ50 transcript variants were used to assess mRNA expression following C-terminal GFP-tagged lamin C or lamin AΔ10 or N-terminal GFP-tagged lamin AΔ50 (Fig. 1B). Results indicate that mRNA expression in MCF7 is increased following transfection.

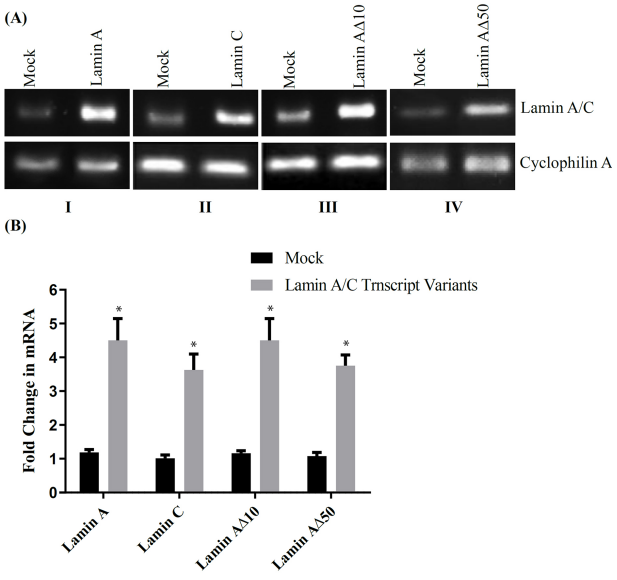

Fig. 1. mRNA Expression and Normalized Fold Change following Upregulation of Lamin A/C Transcript Variants in MCF7 Cell Line. (A) Upregulation of Lamin A/C transcript variants in MCF7 cell line: (I) Total lamin (873 bp); (II) Lamin C (178 bp); (III) Lamin AΔ10 (131 bp) and (IV) Lamin AΔ50 (123 bp) following MCF7 transfection. Cyclophilin A (94 bp) was used as a house keeping gene for gel loading normalization. (B) Cyclophilin A normalized fold change (FC) in mRNA expression following transfection of MCF7 with one of the four lamin A/C transcript variants. Results are presented as mean ± SE; *: $p <$ 0.001 when compared to Mock transfected MCF7; n = 3, unpaired t-test.

3.2 Cellular Distribution of Lamin A/C Transcript Variant Proteins by Confocal Microscopy

MCF7 cell line transfected with pCMV6-AC-GFP expressing chimeric lamin A-GFP-tagged or lamin C-GFP tagged proteins fluorescence showed diffused fluorescent patterns and a perinuclear localization and incorporation into the lamina (Fig. 2A,B,D). Data with C-terminal GFP-tagged lamin AΔ10 revealed that lamin AΔ10 is present in the nucleus as aggregates and the formation of prominent nuclear speckles opposite to lamin A, lamin C and lamin AΔ50 (Fig. 2C). No speckles were observed in the cytoplasm. This is consistent with the previous report by Machiels *et al*. [6]. On other hand, N-terminal GFP-tagged lamin AΔ50 in MCF7 cells significantly induced nuclear shape abnormalities as fluorescence showed a perinuclear localization and incorporation into the lamina with disfigurement of the nucleus, characterized by a lobular shape (Fig. 2D).

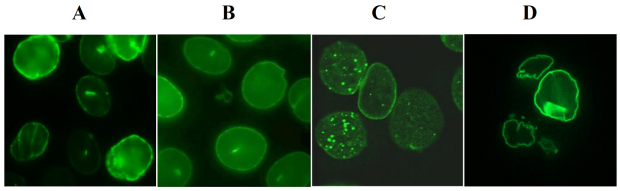

Fig. 2. Confocal microscopy of fixed cultured MCF7 cell line following lamin A/C transcript variants cDNA transfection. Images show the cellular localization of *lamin A/C* gene transcript variant proteins distributions of (A) lamin A-GFP; (B) lamin C-GFP; (C) lamin AΔ10-GFP; and (D) lamin AΔ50-GFP at magnification 100×.

3.3 Activation/Inactivation of Pathways and Upstream Regulators by Lamin A/C Transcript Variants

The 4856 genes that were differentially expressed with lamin A upregulation, 4535 with lamin C, 4877 with lamin AΔ10, and 4562 with lamin AΔ50 genes were analyzed using Ingenuity Pathways Analysis (IPA). DEGs affected by modification of lamin A/C transcript variant were found significantly associated with 71 canonical pathways (Fig. 3 and **Supplementary Table 1**). A comparison analysis was performed to determine the top canonical pathways associated with significant DEGs in lamin A/C transcript variants. The top pathways associated with DEGs that was concordant with both lamin A and lamin AΔ50 upregulation were EIF2 signaling, regulation of eIF4 and p7056K signaling, and mTOR signaling (Fig. 3 and **Supplementary Table 1**). Lamin A and lamin AΔ50 upregulation resulted in pathway patterns that are similar. On the other hand, the top pathways associated with mRNA expres-

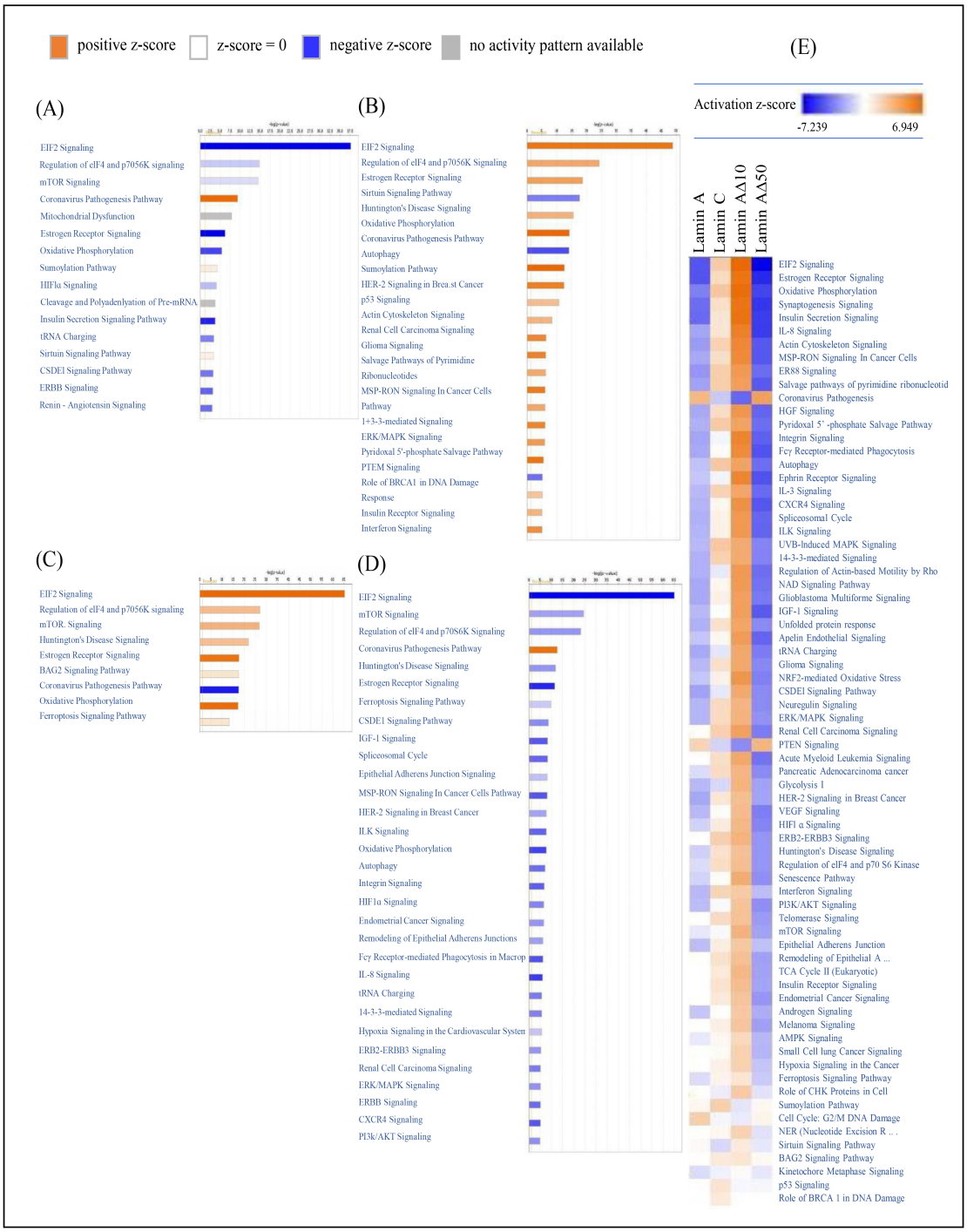

Fig. 3. Top IPA Canonical pathways associated with mRNA expression concordant with the upregulation of Lamin A/C transcript variants. The transcriptional changes associated with the upregulation of different lamin A/C transcript variants (A) Lamin A, (B) Lamin C, (C) Lamin AΔ10, and (D) Lamin AΔ50 in MCF7 cell line compared to mock transfected MCF7 cells were analyzed for canonical pathways using Fisher's exact test with a designated significance threshold of –log *p* value = 1.301 ($p < 0.05$). The results were plotted on a heat map, where the pathways are ranked based on the Z-score, with Z-score $\geq$1 indicating a significant increase (orange), Z-score $\leq$–1 indicating a significant decrease (blue), and undetermined prediction shown in gray. (E) The heat map displays the canonical pathways that were identified following the upregulation of lamin A/C transcript variants in MCF7 cells. The pathways are color-coded based on the Z-score, providing information about the direction and significance of the changes in each pathway. Pathways with Z-score $\geq$1 are shown in orange, indicating a significant increase, while pathways with Z-score $\leq$–1 are shown in blue, indicating a significant decrease. Pathways with undetermined predictions are shown in gray. The yellow straight line represents the designated significant threshold of –log *p* value = 1.301 ($p < 0.05$).

sion patterns that were concordant with both lamin C and lamin AΔ10 upregulation were almost similar and opposite to the activated/inactivated pathway patterns observed following upregulation of lamin A and lamin AΔ50 upregulation (Fig. 3 and **Supplementary Table 1**). Differentially activated/inactivated pathways induced by lamin A versus lamin AΔ50 upregulation were renal cell carcinoma signaling, acute myeloid leukemia signaling, ERB2-ERBB3 signaling, telomerase signaling, remodeling of epithelial adherens junctions, TCA cycle II, insulin receptor signaling, endometrial cancer signaling, melanoma signaling, small cell lung cancer signaling, and hypoxia signaling in the cardiovascular system. Similarly, differentially activated/inactivated pathways induced by lamin C versus lamin AΔ10 upregulation were identified using mRNA data including integrin signaling, ephrin receptor signaling, regulation of actin-based motility by Rho, tRNA charging, CSDE1 signaling pathway, glycolysis I, PI3K/AKT signaling, mTOR signaling, androgen signaling, role of CHK Proteins in cell cycle checkpoint control, and Sumoylation (**Supplementary Table 1**).

The Ingenuity Knowledge base provides tools for upstream regulator analysis based on prior knowledge of expected effects between transcriptional regulators and their target genes [18]. The analysis was conducted using a threshold of p value < 0.05 and activation Z-score ($\geq$2 or $\leq$–2). The results revealed a total of 109, 198, 924, and 979 upstream molecules that were observed following the upregulation of lamin A, lamin C, lamin AΔ10, and lamin AΔ50, respectively, as shown in **Supplementary Tables 2–6**. The top upstream regulators were selected based on the activation Z-score ($\geq$2 or $\leq$–2), and included regulators such as mTOR (mammalian target of rapamycin) and MYC proto-oncogene (MYC). Specifically, in the case of lamin A and lamin AΔ50 upregulation, La ribonucleoprotein domain family member 1 (LARP1) and torin 1 (ATP-competitive inhibitor of mTOR kinase) were found to be activated, while MYC was inactivated. On the other hand, lamin C and lamin AΔ10 upregulation resulted in the activation of MYC and inactivation of LARP1 and torin 1 (Table 2). These findings suggest that lamin A, lamin C, lamin AΔ10, and lamin AΔ50 may have distinct upstream regulators that are involved in regulating the differential expression of genes and that these regulators, including mTOR and MYC, may play important roles in the molecular and cellular functions associated with the transcriptional changes induced by lamin A/C transcript variants in MCF7 cells.

While both lamin C or lamin AΔ10 upregulation were associated with a predicted activation of increased carcinogenesis, cell survival, growth, cell viability, cell proliferation, cell migration, cell invasion, metastasis, and DNA repair and a predicted inactivation of cell death (apoptosis and necrosis), lamin AΔ10 upregulation was associated with a more predicted carcinogenic phenotype when compared with lamin C upregulation (Fig. 4, **Supplementary Table 7**). On the other hand, lamin A or lamin AΔ50 upregulation were associated with a predicted activation of increased cell death (apoptosis and necrosis) and inactivation of carcinogenesis, cell growth, cell viability, cell proliferation, cell migration, cell invasion, and metastasis and was associated with predicted inactivation of glycolysis and mitochondrial respiration. Lamin C upregulation resulted in activation of glycolysis with concomitant inhibition of mitochondrial respiration while lamin AΔ10 upregulation, was associated with increased glycolysis and mitochondrial respiration.

3.4 Anti-Apoptotic and Anti-Senescence Effects of Lamin AΔ10

The findings from the Ion Torrent RNA-Seq data and subsequent experiments suggest that lamin AΔ10 has anti-apoptotic and anti-senescence effects in MCF7 cells. When lamin AΔ10 cDNA was upregulated in MCF7 cells, it resulted in resistance to Doxorubicin, a chemotherapy drug commonly used to induce apoptosis in cancer cells (Fig. 5A). Comparison of mRNA expression levels of lamin A/C transcript variants in MCF7 cells and MCF7/ADR cells (Doxorubicin-resistant) showed higher levels of lamin A/C transcript variants in MCF7/ADR cells, except for lamin AΔ50 which was not detected (Fig. 5B).

Further experiments involved transfecting MCF7/ADR cells with siRNA targeting lamin A/C exon 1 or exon 10, while control cells were transfected with siRNA with limited homology to any known sequences in humans. Transfection with lamin A/C exon 1 siRNA, which targeted the expression of all four transcript variants of lamin A/C, resulted in lower cell survival rate when compared to mock transfected MCF7/ADR cells following incubation with Doxorubicin (Fig. 5C). On the other hand, transfection with lamin A/C exon 10 siRNA, which targeted the expression of lamin A, lamin C, and lamin AΔ50, displayed resistance to Doxorubicin (Fig. 5C).

Furthermore, staining for senescence-associated β-galactosidase (SA-β-Gal), a marker of cellular senescence, showed that lamin A/C exon 1 siRNA transfected MCF7/ADR cells were positive for SA-β-Gal even in the absence of Doxorubicin, and the intensity and percentage of positive cells increased significantly in the presence of Doxorubicin (Fig. 5D). In contrast, lamin A/C exon 10 siRNA transfected MCF7/ADR cells had low SA-β-Gal staining even at high doses of Doxorubicin, indicating a potential anti-senescence role for lamin AΔ10 (Fig. 5D). Taken together, these findings suggest that lamin AΔ10 has anti-apoptotic and anti-senescence effects in MCF7 cells, potentially contributing to resistance to Doxorubicin-induced apoptosis and senescence. Further research may be warranted to elucidate the molecular mechanisms underlying these effects and explore the potential therapeutic implications of lamin AΔ10 in cancer treatment.

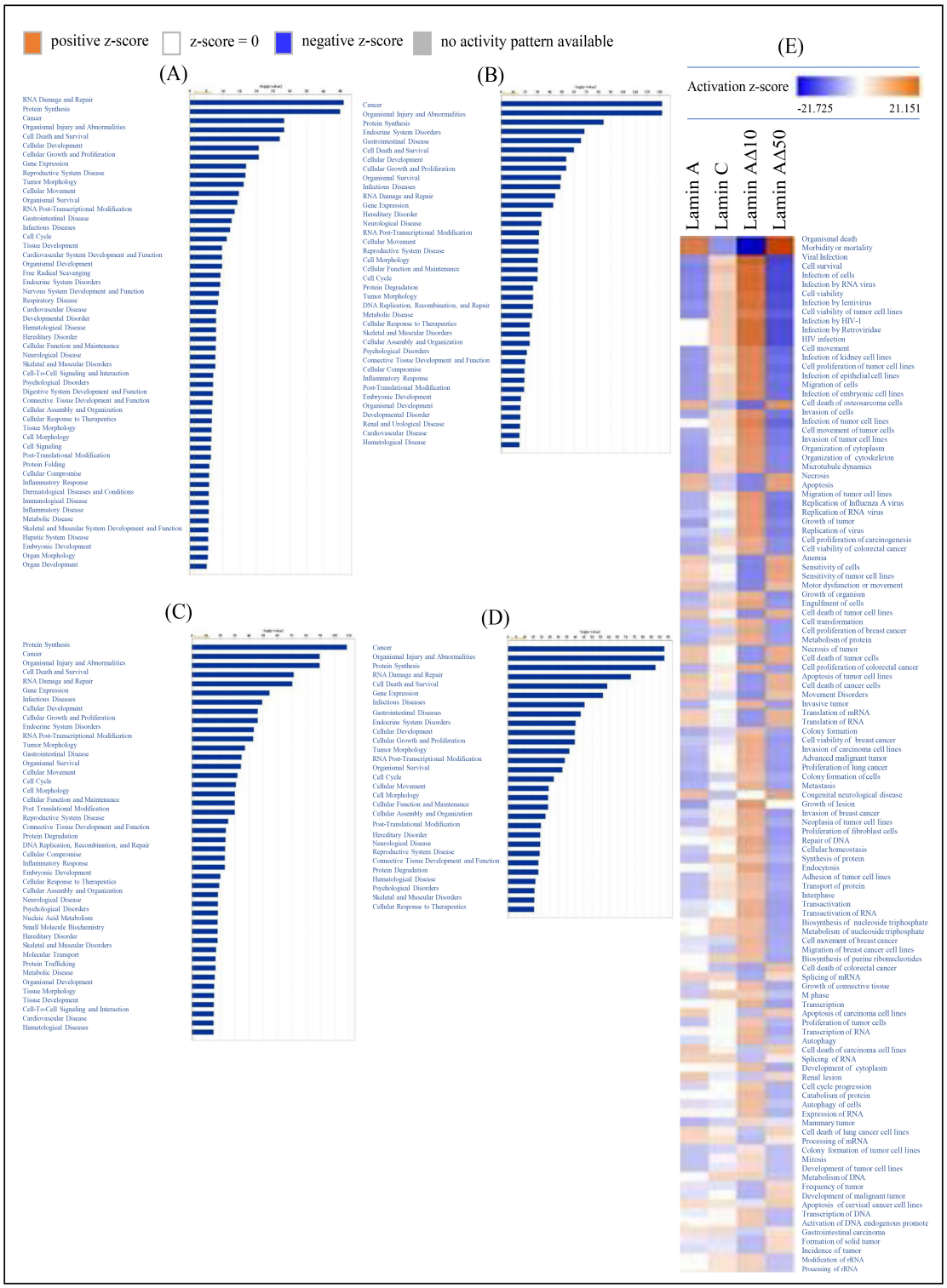

Fig. 4. Molecular and cellular functions, generated through the use of QIAGEN's Ingenuity Pathway Analysis. Molecular and cellular functions associated with transcriptional changes following upregulation of either: (A) Lamin A; (B) Lamin C; (C) Lamin AΔ10; (D) Lamin AΔ50 transcript variants in MCF7 cell line when compared to mock transfected MCF7 cells. The biological functions are ranked by the negative log of the *p* value using Fisher's exact test, right-tailed. The yellow straight line represents the designated significant threshold –log *p* value ($p < 0.05$); (E) A heat map displaying the molecular and cellular functions associated with altered mRNA transcription following upregulation of one of the four lamin A/C transcript variants in MCF7 cell line when compared to mock transfected MCF7 cells.

Table 1. RT-qPCR Primer sequences used.

Primer	Sense (5′→3′)	Anti Sense (5′→3′)	Probe (5′→3′)	Accession Number
Total Lamin A/C	TGAGCAAAGTGCGTGAGGAG	GCTGCGAGGTAGGGCTGG	CGCTGAGTACAACCT	NM_170707.3
Lamin C	GTGGAAGGCACAGAACACCT	GCGGCGGCTACCACTCAC	AGATGACCTGCTCCATCACC	NM_005572.3
Lamin AΔ10	AACTCCACTGGGGAAGGCTCC	GCTCCTGAGCCGCTGGCAGA	AGTACAACCTGCGCTCGCGC	NM_170708.3
Lamin AΔ50	ACTGCAGCAGCTCGGGG	TCTGGGGGCTCTGGGC	AGCATCATGTAATCTGGGACCT	NM_001282626.1
Cyclophilin A (CyPA)	CCCACCGTGTTCTTCGACAT	TTTCTGCTGTCTTTGGGACCTT	ACAGCTCAAAGGAGACGCGGCCCA	NM_021130.5

Table 2. Top upstream regulators of Lamin A/C transcript variants transcriptome.

Upstream regulators	Predicted activation Z-score	*p* value of overlap	Predicted activation
(LARP1), HGNC:29531	5.997	4.92×10^{-36}	Activated
Torin 1	2.906	1.92×10^{-33}	Activated
MYC, HGNC:7553	–6.541	2.90×10^{-35}	Inhibited
LARP1, HGNC:29531	–3.13		Inhibited
Torin 1	–1.333		No Effect
MYC	0.811		No Effect
LARP1, HGNC:29531	–8.714	1.28×10^{-66}	Inhibited
Torin 1	–5.979	2.48×10^{-69}	Inhibited
MYC	10.806	1.87×10^{-70}	Activated
LARP1, HGNC:29531	8.657	3.31×10^{-71}	Activated
Torin 1	4.763	1.55×10^{-56}	Activated
MYC	–9.493	3.66×10^{-65}	Inhibited

4. Discussion

Lamins are intermediate filament proteins located in the nucleus that interact with both chromatin and the cytoskeleton of the cell [9]. The levels of different transcript variants of lamin A/C change during normal development and in diseases, but the exact mechanism behind this differential expression is not yet fully understood. It is believed that this variation in expression could be attributed to altered mRNA splicing, reduced mRNA stability, reduced protein stability, or reduced translation efficiency [19]. To better comprehend the role of lamins and their contribution to diseases, it is crucial to measure their relative abundances in both normal and cancerous tissues. A previous study investigating the expression levels of lamin A, lamin C, lamin AΔ10, and lamin AΔ50 mRNA in 47 different normal tissues and organs revealed that the expression of lamin A/C transcript variants varies across tissues [14]. Most normal human tissues primarily express lamin A and lamin C, with lamin AΔ10 and lamin AΔ50 being less abundant [6,14]. Notably, the placenta exhibits very high expression of lamin AΔ10, followed by seminal vesicles, while the kidney shows the least mRNA expression. Lamin A expression is often reduced or absent in less differentiated and highly proliferating cells, such as undifferentiated embryonic stem (ES) cells [20–23], suggesting that lamin A may be implicated in the maintenance of cellular differentiation [23–25].

Mutations in the Lamin A/C gene can result in various laminopathies, including muscular dystrophy, neuropathy, diabetes, lipodystrophy [26], Hutchinson-Gilford Progeria Syndrome (HGPS) affecting children, and Werner syndrome in adults [27,28]. Additionally, alterations in the composition of nuclear lamins have been implicated in malignant transformation, tumor propagation, and progression. Loss of lamin A/C has been reported in lung cancer [29], breast cancer [30,31], colon cancer [30,32], colonic and gastric adenocarcinoma [30], primary gastric carcinoma [33], basal cell skin carcinoma [34], leukemia [35], ovarian cancer [36,37] and prostate cancer [38,39]. However, there are conflicting findings as up-regulation of lamin A/C has also been observed in breast cancer [14], prostate cancer [40,41], skin cancer [42], ovarian serous cancer [43], and colorectal cancer [44,45]. In fact, increased expression of lamin A/C in colorectal cancer has been shown to increase invasiveness and cell motility [45,46], while increased lamin A/C in prostate cancer cells has been linked to stimulation of cell growth, colony formation, migration, and invasion [41]. Understanding the mechanisms underlying these diseases is challenging, as most studies have detected LMNA/C gene transcript variants as one protein with one function, without considering each variant as a separate protein with different levels of disease involvement and associated laminopathies (Table 3, Ref. [29–39,42–45]). Moreover, the methods used for studying lamins, such as semi-quantitative and insensitive Western blotting (WB) and immunohistochemistry (IHC), have limitations. For instance, there is currently no specific antibody for lamin AΔ10, and attempts to generate one have failed [6]. Additionally, lamin AΔ50 is low abundant in normal cells and generally undetectable with current methods [47–49] and can only be detected in HGPS cells [10,27,28,50]. The recent development of specific methods for the measurement of the four transcript variants at the mRNA level [14] and protein level [13,51] could provide valuable insights into the role of lamin A/C transcript variants in different path.

Table 3. Lamin A/C expression in different cancer types.

Type of cancer	Lamin-A/C
Lung cancer [29]	↓
Breast Cancer [30,31]	↓
Colon cancer [30,32]	↓
Colorectal cancer [44,45]	↑
Colonic and gastric adenocarcinoma [30]	↓
Primary gastric carcinoma [33]	↓
Basal cell skin carcinoma [34]	↓
Skin cancer [42]	↑
Leukemia [35]	↓
Ovarian serous cancer [43]	↑
Ovarian cancer [36,37]	↓
Prostate cancer [38,39]	↓

The IPA (Ingenuity Pathway Analysis) is used to predict activity patterns of pathways based on curated literature [18]. The comparison analysis showed that lamin A and lamin AΔ50 transcript variants had similar patterns of activated/inactivated pathways that were opposite to the patterns observed with lamin C and lamin AΔ10. However, there were also differences in the activated/inactivated pathways induced by lamin A versus lamin AΔ50 or lamin C versus lamin AΔ10. Upregulation of lamin A or lamin AΔ50 in MCF7 cell line activated LARP1 and torin 1 (ATP-competitive inhibitor of mTOR kinase) while inactivating MYC. Lamin C or lamin AΔ10 upregulation, on the other hand, activated MYC and inactivated LARP1 and torin 1. LARP1 functions as a key effecter and regulator for mTORC1 by repressing the translation of TOP mRNAs downstream of mTORC1 and thus regulates cell death and translation of mRNA [52]. Torin 1 is able to effectively block phosphorylation of mTORC1 and mTORC2 and thus induces autophagy, as inhibition of mTOR mimics cellular starvation by blocking signals required for cell growth, proliferation, reduce protein synthesis and arrest cell cycle progression in G1 [53,54]. The *MYC* gene (MYC Proto-Oncogene, BHLH Transcription Factor) belongs to an oncogene encoding a nuclear protein that is involved in cell cycle regulation. The *MYC* gene family promotes cell proliferation, immortalization, dedifferentiation and transformation. This suggests that Lamin C or lamin AΔ10 upregulation are associated with carcinogenic phenotype

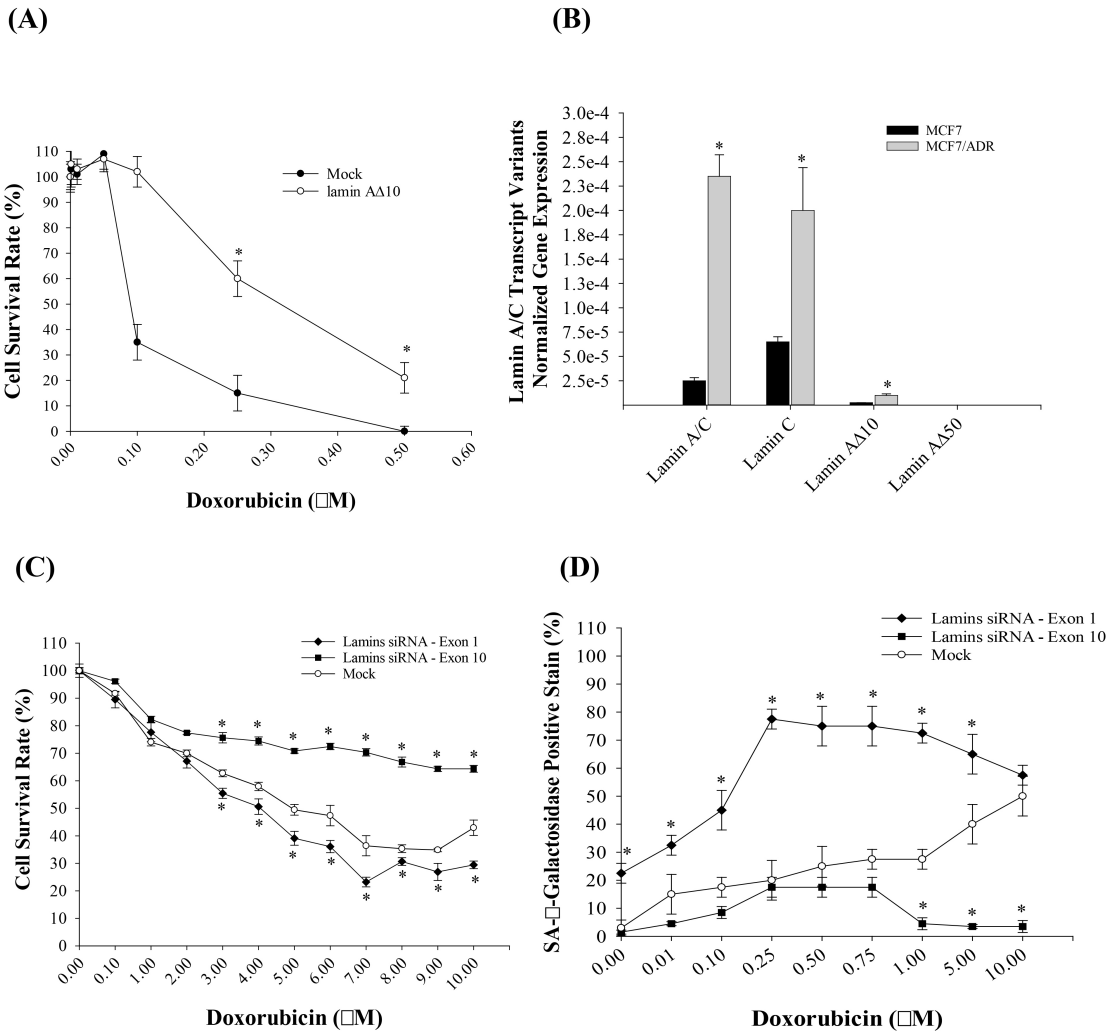

Fig. 5. Lamin A/C transcript variants expression in MCF7/ADR cell line and the effect of Lamin AΔ10 inhibition on cell survival and Senescence-associated beta-galactosidase (SA-β-Gal stain). MCF7 cell survival following lamin AΔ10 cDNA upregulation. (B) Lamin A/R transcript variants mRNA expression in MCF7 and MCF7/ADR as measured by RT-qPCR utilizing lamin A/C transcript variants specific primers except Lamin A. Results demonstrate significant increase of total lamin, lamin C and lamin Δ10 mRNA. (C) MCF7/ADR transfection with plasmids expressing lamin A/C exon 1 siRNA resulted in increased doxorubicin sensitivity while targeting lamin A/C exon 10 resulted in increased doxorubicin resistance. Cells were incubated with doxorubicin for 48 hours. (D) SA-β-Gal stain was absent in most of mock transfected MCF7/ADR cells or lamin A/C siRNA against exon 10. Challenging these cells with different concentrations of doxorubicin for 48 hours increased the percentage of cells stained with SA-β-Gal except for MCF7/ADR cells transfected with lamin A/C exon 1 siRNA . Results are presented as mean ± SE; *: $p < 0.001$ when compared to Mock transfected MCF7; n = 3, unpaired *t*-test.

by modulating MYC, LARP1 and torin 1 pathways. This phenotype is associated with a predicted activation of increased carcinogenesis, cell survival, growth, cell viability, cell proliferation, cell migration, cell invasion, metastasis, and DNA repair and a predicted inactivation of cell death (apoptosis and necrosis) observed following upregulation of Lamin C or lamin AΔ10. Lamin A or lamin AΔ50 upregulation, on the other hand, were associated with a predicted activation of increased cell death (apoptosis and necrosis) and inactivation of carcinogenesis was associated with predicted inactivation of glycolysis and mitochondrial respiration suggesting that the energy source relies mainly on the metabolism of glucose which is characterized by increased rates of glycolysis and lactate production (Warburg effect) even in the presence of oxygen [55]. Interestingly, lamin C upregulation resulted in activation of glycolysis with concomitant inhibition of mitochondrial respiration while lamin AΔ10 upregulation was associated with increased glycolysis and mitochondrial respiration. The increased glycolysis and mitochondrial respiration, observed with lamin AΔ10 upregulation, is consistent with dysregulated metabolism which has been linked to increased tumor

aggressiveness and treatment resistance [56]. These data support the notion that lamin AΔ10 upregulation is associated with a more carcinogenic and aggressive tumor phenotype.

The effect of LMNA inhibition on cell survival was examined on MCF-7/ADR. Establishing a specific siRNA for each transcript variant is a tedious work and the possibility of off target is almost always present. Off-target effect is mainly induced by the unintended cross hybridization between siRNAs and endogenous RNA sequences, other than the ones specifically targeted. This can obscure the aimed functional interpretation in gene silencing experiments, and must be avoided as much as possible [57]. Targeting exon 1 reduced doxorubicin chemoresistance as a result of inhibition of lamin A, lamin C, lamin AΔ10, and lamin AΔ50. Targeting exon 10 results in the inhibition of all lamin A/C transcript variants except lamin AΔ10 and this increased doxorubicin resistance. Inhibition of all lamin A/C transcript variants resulted in an increased SA-β-gal stain in MCF7/ADR following doxorubicin addition while targeting exon 10 of lamin A/C resulted in decreased stain of SA-β-gal stain. These data strongly suggest that lamin AΔ10 regulates senescence and inhibition of lamin AΔ10 results in increased senescence and apoptosis. Interestingly, lamins are one of the first nuclear targets cleaved during apoptosis. The A type Lamins cleavage by caspase-6 is an important step in the nuclear apoptotic process and concurs with their total disintegration from the nuclear lamina during apoptosis [58–62]. The caspase-6 cleavage site (VEID) for A type lamins [59] is in exon 4 and thus would inactivate all lamin A/C transcript variants. This represents a plausible hypothesis that lamin AΔ10 cleavage is essential for the nuclear apoptotic process and requires further investigation.

MCF7 cells transfected with lamin AΔ10-GFP construct expressing GFP at the C-terminal in this study showed a stable expression of the chimeric proteins. In the majority of transfected cells, lamin Δ10-GFP revealed prominent nuclear speckles. This is consistent with the Machiels *et al*. [6] study which was first to report the expression of lamin AΔ10 in several cell lines. Machiels *et al*. [6] study detected an abnormal localization of lamin A as nuclear aggregates in the adenocarcinoma cell line GLC-A1 using immunofluorescence microscopy when stained with an antibody directed to lamin A. Lamin A is synthesized as prelamin A, with a C-terminal CaaX motif, and then undergoes a series of posttranslational modifications in the nucleus (farnesylation, aaX cleavage and carboxylmethylation, and cleavage by Zmpste24) [63]. Since lamin C lacks a CaaX motif, it is not modified. However, the processing enzymes have been shown to reside either in the cytosol (farnesyltransferase), or are endoplasmic reticulum (ER) membrane proteins (Zmpste24, Rce1, and ICMT) [63]. Both prelamin A and lamin AΔ50 have been shown to undergo these posttranslational modifications whereas lamin AΔ10 has not been investigated. However, lamin Δ10 has been demonstrated to be localized to the nuclear membrane (perinuclear localization) suggesting that it does undergo posttranslational modifications in the *Chinese Hamster Ovary* (*CHO-K1*) *cells*. The majority of transfected cells with one of the three lamin A/C transcript variants-GFP chimera (lamin A. lamin C, and lamin AΔ10) revealed an extensive collection of branching intra- and transnuclear tubular structures [64]. Interestingly, blocking farnesylation, the first step of CaaX processing, causes nucleoplasmic accumulation of completely unprocessed prelamin A [65,66] and inhibition of farnesylation of lamin AΔ50 prevents the characteristic nuclear blebbing of Hutchinson-Gilford progeria syndrome [67]. However, our data with C-terminal GFP-tagged lamin AΔ10 suggest that unprocessed lamin AΔ10 is present in the nucleus as aggregates opposite to unprocessed lamin A and lamin AΔ50 which show a diffused fluorescent pattern. Overexpression of C-terminal GFP-tagged lamin AΔ10 in MCF7 cells resulted in the formation of prominent nuclear speckles and no speckles were observed in the cytoplasm. Additionally, no speckles were formed when cells were transfected with C-terminal GFP-tagged lamin A, or lamin C or lamin AΔ50. The role of the aggregation and nuclear localization of lamin AΔ10 in the nucleus is unknown and needs further investigation.

5. Conclusions

Dissecting the signal transduction pathways of lamin A/C transcript variants is crucial to identify the mechanisms for the different pathophysiological processes associated with laminopathies. Lamin C and lamin AΔ10 upregulation are associated with a predicted increased carcinogenesis, cell survival, growth, cell viability, cell proliferation, cell migration, cell invasion, metastasis, and DNA repair and a predicted inactivation of cell death by modulating MYC, LARP1 and torin 1 pathways. However, lamin AΔ10 upregulation is associated with a more carcinogenic and aggressive tumor phenotype as compared to lamin C. Lamin A or lamin AΔ50 upregulation is associated with a predicted activation of increased cell death and inactivation of carcinogenesis. Lamin AΔ10 only form prominent nuclear speckles and aggregates rather than the conventional perinuclear localization observed with other lamin A/C transcript variants.

Abbreviations

HGPS, Hutchinson-Gilford progeria syndrome; MEM, Eagle minimum essential médium; CypA, Cyclophilin A; MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide); SA-β-gal, Senescence-associated beta-galactosidase; NGS, Next-Generation Sequencing; IPA, Ingenuity Pathway Analysis; LARP1, La ribonucleoprotein domain family member 1; MYC, MYC proto-oncogene; siRNA, small interfering RNA; GFP, green fluorescent protein.

Availability of Data and Materials

Materials used in this study can be obtained from the corresponding authors upon request.

Author Contributions

Conceptualization, RF, AA; methodology, LB, MZ, JH, MAA, WA; formal analysis, MAA, JH; resources, AA, RF; data curation, JH, MZ; writing—original draft preparation, LB, JH; writing—review and editing, RF, AA; visualization, MAA; supervision, RF, AA; project administration, RF; funding acquisition, RF. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

The study was approved by the Institutional Review Board (IRB) of Ministry of National Guard Health Affairs (MNGHA, Protocol Code RC10/113).

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2806113.

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