TGF-β controls stromal telomere length through epigenetic modifications

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Abstract
Telomere length is primarily controlled by the enzyme telomerase, but being chromatin structures, telomeres undergo epigenetic regulation for their maintenance and function. Altered telomere length among cancer cells combined with shorter telomere length in cancer-associated stromal cells, strongly implicated with progression to prostate cancer metastasis and cancer death and providing a novel target for therapeutics. Transforming growth factor-β (TGF-β) signaling pathways are well-recognized for their role in stromal-epithelial interactions responsible for prostate androgen responsiveness, promoting tumorigenesis. However, the underlying mechanism remains unclear. We sought to establish a role for TGF-β in the regulation of telomere length in mouse and human prostate fibroblast. Polymerase chain reaction (PCR)-based telomere length measuring methods are widely used due to their repeatability and reproducibility. Using real-time RT-PCR-based telomere length measuring method, we identified that TGF-beta regulates telomere length via increased expression of histone methyltransferase, Suv39h1, which in turn affected histone methylation levels at the telomeric ends. Moreover, treatment of DAPT and non-steroidal antiandrogen bicalutamide demonstrated that notch and androgen signaling co-operated with TGF-β in regulating stromal telomere length. Telomere variation in tumor cells and non-tumor cells within the tumor microenvironment greatly facilitates the clinical assessment of prostate cancer; therefore, understanding stromal telomere length regulation mechanism will hold significant prospects for cancer treatment, diagnosis, and prognosis.

Keywords Telomere length · Epigenetics · TGF-β · Tumor microenvironment · Cancer

Introduction
Telomeres are protective “caps” on eukaryotic chromosomes, composed of long tandem arrays of double stranded TTAGGG repeats bound by specialized array of specialized proteins (Martinez and Blasco 2017; Morin 1989). Normal cells rely on telomeres to maintain genomic integrity as the progressive shortening of telomere during successive cell divisions induces chromosomal instability. In many cancers, telomere length is maintained by enzyme telomerase; therefore, telomere length and telomerase activity are integral to cancer initiation and the survival of tumors (Harley et al. 1990; Morin 1989; Nandakumar and Cech 2013). Telomeres are chromatin structures that respond to different epigenetic modifications, including DNA methylation, histone modification, chromatin remodeling, and RNAi in mediating DNA condensation and ultimately length (Fojtova and Fajkus 2014; Jezek and Green 2019). There is evidence suggesting that telomeres can be either enriched or depleted of heterochromatin marks such as di- and trimethylation of lysine9...
of histone H3 (H3K9) that recruit heterochromatin protein 1 (HP1) (Cubiles et al. 2018; Vacik et al. 2005; Vaquero-Sedas and Vega-Palas 2019).

This epigenetic heterogeneity extends into the telomeres of both epithelial and fibroblastic cells (Shay 2013). Understanding the mechanism of telomere length regulation is a potential target of antituumor therapy. The shortening of telomere is a process of cell death that can serve as a hallmark of cellular aging (Hu et al. 2018). Prostate cancer (PCa) is one of the most common age-related diseases, therefore, alterations in telomere length are among the earliest events seen in this type of cancer (Graham and Meeker 2017). PCa cells have robust telomerase activity to maintain telomere length, as opposed to that seen in normal primary prostate epithelial cells with negligible telomerase activity (Graham and Meeker 2017; Heaphy and Meeker 2011; Heaphy et al. 2013). Other mechanisms of maintaining telomere length, such as alternative lengthening of telomeres, are also reported in many other cancers (Agrawal et al. 2021; Sharma et al. 2021), including metastatic PCAs (Graham and Meeker 2017; Heaphy et al. 2013). Telomere length measurements of prostate tumor specimens obtained at the time of surgery or taken at the time of biopsy were identified as promising biomarkers of the outcome (Baena-Del Valle et al. 2018; Mehrez et al. 2019).

Transforming Growth Factor β (TGF-β) is a pleiotropic cytokine (Herpin et al. 2004) that play an important role in promoting tumor, either by directly promoting epithelial-mesenchymal transition (EMT), thereby enhancing the migration, invasion, infiltration, and extravasation in tumor cells (Massague 2012), or indirectly by inducing abnormal tumor microenvironment (TME), such as activating cancer-associated fibroblasts (CAF), promoting angiogenesis as well as inhibiting anti-tumor immune response thereby promoting tumor metastasis. An abnormal TME is regarded as a critical event in tumor initiation and progression by modulating epithelial–stroma interactions which increase the probability of a preneoplastic lesion to turn in the malignant cell (Bhowmick et al. 2004; Placencio et al. 2008; Kiskowski et al. 2011). In prostate TME, apart from TGF-β, androgen and Notch signaling pathways are also critically involved in tumor–stroma interaction which helps in determining the differentiation states of the prostate. TGF-β is known to limit cell proliferation and induce cell senescence, and both phenomena were found to be regulated by telomeres and telomerase (Li et al. 2006). Moreover, interruption of TGF-β autocrine actions was linked with high telomerase activity in breast cancer; on the contrary, restoring autocrine TGF-β activity decreases telomerase activity in colon cancer (Yang et al. 2001). The present study was undertaken to characterize the actions of TGF-β, and its associated factor in the regulation of stroma telomere length in the prostate stroma as stromal telomere length serves as critical prognostic markers for metastasis and death in prostate cancer (Heaphy et al. 2013).

Androgen receptor signaling inhibitors (ARSIIs) are known to dramatically improve the treatment of prostate cancer and found to be useful in improving overall survival (OS) for all types of prostate cancer (Asif and Teply 2021). Interestingly ARSIIs also known to promote telomere shortening through the inhibition of telomerase expression in PCa (Liu et al. 2010), we speculated a possible role of telomere length in prostate cancer progression, the links to Notch and TGF-β signaling on telomere length. Evidence is accumulating about involvement of Notch signaling in regulating telomere length in endothelial cells, which support the narration that ARSI therapeutic resistance occurring through signaling in prostatic stromal fibroblasts (Liu et al. 2010; Heaphy et al. 2013). Juxtaprine signaling involving Notch heterodimeric transmembrane receptors binds transmembrane ligands, Delta-like proteins (Delta1 and 3), and Jagged (1 and 2). Since both receptors and ligands are membrane-bound, cell–cell contact is necessary to trigger receptor/ligand activation of γ-secretase to cleave the Notch receptor intracellular domain (NICD) proteolytically. The release of NICD enables its translocation to the nucleus, contributing to the assembly of a transcriptional complex that initiates Notch downstream targets (Jarriault et al. 1995; Nam et al. 2003; Kopan and Ilagan 2009). TGF-β signaling in prostatic fibroblasts can drive tumor growth and ARSI resistance (Ao et al. 2006; Bhowmick et al. 2004; Bhowmick and Moses 2005). Interestingly, the frequency of epigenetic silencing of TGF-β type II receptor gene (Tgfr2) in PCa associated fibroblastic cells is not observed in PCa epithelia (Banerjee et al. 2014). These findings in patients were phenocopied in transgenic mouse models with a conditional knockout of Tgfr2 in a subset of stromal fibroblasts (Tgfr2-KO) (Bhowmick et al. 2004; Jackson et al. 2012). Telomere shortening in stromal fibroblasts is associated with PCa metastatic progression and mortality (Graham and Meeker 2017; Heaphy et al. 2013; Laberthonniere et al. 2019). The most significant feature of short telomeres, either in epithelial or fibroblastic cells, is acquiring a senescence-associated secretory phenotype (SASP), permissive for cancer progression (Coppe et al. 2010). Cancer epithelial and stromal interaction was known to promote carcinoma-associated fibroblasts (CAF) via notch signaling Notch signaling in breast cancer (Strell et al. 2019). Even in PCa, Notch signaling is found expressed in CAF and found to play a tumorigenic role (Orr et al. 2013); however, the implications of this are still unclear.

This study aimed to identify the involvement of TGF-β and its associated factor, which is involved in telomere length regulation. Our results identified that loss of TGF-β expression in prostate stromal fibroblast led to telomere shortening, an effect mediated by upregulating histone.
methyltransferase, Suv39h1 followed by HP1 (heterochromatin protein 1) recruitment at the telomere. Moreover, notch and androgen signaling also co-operate with TGF-β in regulating stromal telomere length, as demonstrated by inhibitors such as γ-secretase inhibitor DAPT, which inhibits Notch signaling and bicalutamide, a non-steroidal anti-androgen used in the treatment of prostate cancer. Our study identifies a link between that notch and androgen signaling, which co-operated with TGF-β in regulating stromal telomere length in prostate cancer.

Materials and methods

Animals and cultured cells

Primary mouse prostate stromal cell cultures were generated from 6 to 8-week-old Tgfbr2floxE2/floxE2 (WT) and Tgfbr2fspKO (TKO) mice as described before (Banerjee et al. 2014). CAF (Cancer Associated Fibroblasts) and NAF (Normal Associated Fibroblasts) cells were similarly developed from fresh human prostatectomy tissues (Banerjee et al. 2014). Mouse and human stromal primary cultures were used in the first ten passages only. All cultures were grown in a humidified 5% CO₂ environment at 37 °C. Mouse studies were approved and performed by approved Cedars-Sinai Animal Care and Use Committee protocol.

Telomere length quantification by real-time quantitative PCR (qPCR)

Telomere length was analyzed as described before (Cawthon 2002). Briefly, genomic DNA was isolated from fibroblasts using DNAeasy Blood and Tissue Mini Kit (Qiagen; Valencia, CA) as described in the manufacturer’s protocol and qPCR was performed. PCR conditions and primers are as described by Cawthon (2002), where parallel pre-amplification telomere variable repeat region (TTAA GG) and a single-copy gene (36B4) for subsequent PCR threshold cycle value (Ct), measured against standards of known copy number (Cawthon 2002). The ratio of telomere-repeat and 36B4 is directly proportional to individual relative telomere length. All qPCR reactions were performed on the 7500 Real-Time PCR System (Applied Biosystems; Foster City, CA).

Methylation-specific PCR (MS-PCR)

MS-PCR was performed as described before (Mishra et al. 2019). Bisulfite treatment was performed on DNA isolated from wild type and acrolein treated cultured mouse stromal fibroblasts using the EZ DNA methylation-Gold kit (Zymo Research, Irvine, CA) according to the vendor’s recommendations. Bisulfite converted DNA was amplified by Methylation Specific-PCR. Sequences of Tgfr2 for the unmethylation reaction were 5’-ttgaaagttggttaaagtttttgga-3’ (forward) and 5’-aagaaagttcctctctcctcaca-3’ (reverse), and primer sequences for the methylated reaction were 5’-gaagtggttaagttggtttaaagtttttgga-3’ (forward) and 5’-aagaaagttcctctctcctcaca-3’ (reverse) as described before (Zhang et al. 2004).

Telomere ChIP

ChIP assay was performed using the Zymo-Spin ChIP kit (Zymo Research; Irvine, CA) following the manufacturer’s protocol as described before (Mishra et al., 2019). The antibodies used for ChIP assay were anti-H3K9me3 from Abcam (Cat#ab8898) and anti-HP1α (EMD Millipore, Cat#05-689). PCR was used to analyze the occupancy of H3K9me3 and HP1 on the TERT gene promoter with the primer sequences that were previously described (O’Callaghan and Fenech 2011). DAPT (N-[(N-(3,5-Difluorophenacetyl)-l-alanyl]–l-phenylglycine t-butyler; Cat#2634, from Tocris Bioscience, Minneapolis, MN) was used to inhibit Notch signaling.

Western blot analysis

Western blots performed with 4–12% SDS–polyacrylamide gels (Mishra et al. 2019). In brief, the following electrophoresis gels were transferred to PVDF membrane (BioRad) in a transfer buffer (25 mM Tris; 200 mM glycine; 20% methanol v/v). Membranes were blocked and subsequently incubated with primary and secondary antibodies in phosphate-buffered saline containing 0.1% Tween20 (Sigma) and 5% non-fat dry milk or bovine serum albumin for at least 1 h each. Detection was performed using alkaline phosphatase-conjugated secondary antibodies (Sigma-Aldrich). Anti-Telomerase reverse transcriptase antibody from Abcam (Cat#ab230527), Anti-SUV39H1 (Upstate Millipore; Cat#07-550), anti-H3K9me3 from Abcam (Cat#ab8898), anti-DNMT1 from Abcam (Cat#92453) and β-actin antibody (Santa Cruz Biotechnology) were used for detection. Western blots were visualized using alkaline phosphatase-conjugated secondary antibodies (Sigma-Aldrich). Experiments were repeated in at least three independent experiments, and one of the representative blots was shown. Transfection of siRNA was done by lipofectamine reagent (Invitrogen, Carlsbad, CA) as previously described (Mishra et al. 2019). SUV39H1 gene silencing was performed using a pool of siRNA (sc-38463, Santa Cruz Biotechnology, Santa Cruz CA). Scrambled siRNA was used from the control. SUV39H1 knockdown was validated by qPCR method.
PCR arrays

The RT² Profiler™ PCR Array of Human Telomeres & Telomerase PCR Array (Cat. # PAHS-016ZE-4; Qiagen; Frederick, MD), which detects the expression of 84 essential genes (Listed in Table 1). Fold changes in gene expression relative to control samples were analyzed using the Excel datasheet provided at the Qiagen gene globe website (https://geneglobe.qiagen.com/product-groups/custom-rt2-profiler-pcr-arrays), and volcano plot was created using GraphPad Prism 6 (La Jolla, CA).

Bioinformatics analysis

We used the public database cBioPortal for Cancer Genomics (Cerami et al. 2012; Gao et al. 2013). This portal collects next generation sequencing data from The Cancer Genome Atlas (TCGA) and the International Cancer Genome Consortium (ICGC). We selected Prostate Adenocarcinoma datasets (MSKCC, Cancer Cell 2010) from cBioportal and calculated the Spearman correlation coefficient between SUV39H1 expression and other Notch downstream genes (HEY1, CCND3, and MYC). To analyze the expression profile of shelterin complex genes, UACLAN database (Chandrasheker et al. 2017) were examined in prostate adenocarcinoma (PRAD) using the web-portal UALCAN (http://ualcan.path.uab.edu/index.html). UALCAN analyses are based on TCGA dataset to determine expression, survival analysis, and evaluate promoter DNA methylation. The shelterin complex genes expression profile of PRAD includes 497 tumor tissue samples and 52 non-tumor prostate tissue samples.

Statistical analysis

Statistical analysis of significance was calculated using one-way analysis of variance for multigroup comparisons using GraphPad Prism 6 (La Jolla, CA). To analyze time-dependent telomere length (https://www.socsistatistics.com/tests/anovarepeated/default.aspx), analysis of repeated measure ANOVA was used to perform statistical significance. The results are shown as the mean ± SEM. P values < 0.05 were considered statistically significant.

Results

TGF-β-dependent telomere shortening in mouse prostatic fibroblasts

Telomere length in mouse prostatic fibroblasts was evaluated in response to TGF-β signaling. Genomic DNA (gDNA) was extracted from cultured WT or TKO mouse prostatic fibroblasts. The ratio of telomere-to-single-copy-gene determines relative telomere length. The number of telomere repeats in each sample was correlated with the total number of the single-copy gene, 36B4, in the DNA samples (Fig. 1A). The linear relationship allowed for a simple relative quantitation of the unknowns (Supplementary Fig. 1A, 1B). Subsequently, mouse genomic DNA samples from WT and TKO fibroblast telomere lengths were compared for a causal role of TGF-β on stromal fibroblast. The relative telomere length was significantly shorter in TKO fibroblasts than WT controls (P < 0.001, Fig. 1B). Interestingly, when we compared mRNA expression between WT and TKO fibroblast, the TKO fibroblast showed a significant reduction in expression compared to WT fibroblast (P < 0.001, Fig. 1C). We also evaluated human NAF and CAF and found that CAF had reduced levels compared to NAF (P < 0.001, Fig. 1D). These results suggest that TGF-β regulation has a strong influence on telomere length and transcript level and in line with our previous report where we demonstrated that epigenetic silencing of TGFBR2 in human prostatic CAF, dictates stromal coevolution mediated tumor progression (Banerjee et al. 2014). To evaluate the role of TGFBR2 epigenetic silencing on telomere length in human prostatic fibroblasts, we initially analyzed TGFBR2 promoter methylation status in NAF and CAF. TGFBR2 promoter methylation was more significant in CAF compared to NAF, associated with gene downregulation, as previously reported (Fig. 2A) (Banerjee et al. 2014). Concomitant telomere length analysis of the same cells demonstrated that the CAF had significantly shorter telomeres than NAF (Fig. 2B). To further investigate the effect of TGF-β on methylation, we analyzed protein expression of methylation enzymes and histones, including DNMT1, H3K9me3, and histone methyltransferase (SUV39H1) in wild type mouse fibroblast. Mouse fibroblasts were treated with TGF-β inhibitor, LY36497, over a time course of 12, 24, and 48 h. Western blots demonstrated that mouse fibroblast had elevated SUV39H1 after 24 h of TGF-β signaling inhibition; however, DNMT1 and H3K9me3 protein expression were unaltered over the same time course (Fig. 2C). SUV39H1 protein expression was also upregulated in the TKO prostatic fibroblasts, compared to its control (Supplementary Fig. 2). Accordingly, SUV39H1 was knocked down using a siRNA pool to determine its role in telomere length. We found that SUV39H1 knockdown restored the telomere length of the TKO fibroblasts to near that of WT fibroblasts (Fig. 2D). We next examined the localization of heterochromatin marks, H3K9me3, and HP1 at telomeres. There was an elevated association of both H3K9me3 and HP1 to the telomeric repeat sequence by ChIP analysis in both TKO and WT fibroblasts treated with or without LY36497 (Fig. 2E, F). These results indicated that the loss of TGF-β signaling
| S no. | NCBI Gene ID | RefSeq ID | Symbol | Description |
|-------|--------------|-----------|--------|-------------|
| 1     | 25           | NM_005157 | ABL1   | C-abl oncogene 1, non-receptor tyrosine kinase |
| 2     | 65,057       | NM_022914 | ACD    | Adrenocortical dysplasia homolog (mouse) |
| 3     | 207          | NM_005163 | AKT1   | V-akt murine thymoma viral oncogene homolog 1 |
| 4     | 472          | NM_000051 | ATM    | Ataxia telangiectasia mutated |
| 5     | 509          | NM_005174 | ATP5C1 | ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1 |
| 6     | 596          | NM_000633 | BCL2   | B cell CLL/lymphoma 2 |
| 7     | 641          | NM_000057 | BLM    | Bloom syndrome, RecQ helicase-like |
| 8     | 1017         | NM_001798 | CDK2   | Cyclin-dependent kinase 2 |
| 9     | 1111         | NM_001274 | CHEK1  | CHK1 checkpoint homolog (S. pombe) |
| 10    | 11200        | NM_007194 | CHEK2  | CHK2 checkpoint homolog (S. pombe) |
| 11    | 64858        | NM_022836 | DCLRE1B| DNA cross-link repair 1B |
| 12    | 64,421       | NM_022487 | DCLRE1C| DNA cross-link repair 1C |
| 13    | 1736         | NM_001363 | DKC1   | Dyskeratosis congenita 1, dyskerin |
| 14    | 1950         | NM_001963 | EGF    | Epidermal growth factor |
| 15    | 146,956      | NM_152463 | EME1   | Essential meiotic endonuclease 1 homolog 1 (S. pombe) |
| 16    | 2067         | NM_001983 | ERCC1  | Excision repair cross-complementing rodent repair deficiency, complementation group 1 (includes overlapping antisense sequence) |
| 17    | 2072         | NM_005236 | ERCC4  | Excision repair cross-complementing rodent repair deficiency, complementation group 4 |
| 18    | 54,433       | NM_018983 | GAR1   | GAR1 ribonucleoprotein homolog (yeast) |
| 19    | 8,520        | NM_003642 | HAT1   | Histone acetyltransferase 1 |
| 20    | 3181         | NM_002137 | HNRNPA2B1 | Heterogeneous nuclear ribonucleoprotein A2/B1 |
| 21    | 3184         | NM_002138 | HNRNPD | Heterogeneous nuclear ribonucleoprotein D (AU-rich element RNA binding protein 1, 37 kDa) |
| 22    | 3320         | NM_001017963 | HSP90AA1 | Heat shock protein 90 kDa alpha (cytosolic), class A member 1 |
| 23    | 3305         | NM_005527 | HSPA1L | Heat shock 70 kDa protein 1-like |
| 24    | 3479         | NM_000618 | IGFI   | Insulin-like growth factor 1 (somatomedin C) |
| 25    | 3845         | NM_004985 | KRAS   | V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog |
| 26    | 889          | NM_194454 | KRIT1  | KRIT1, ankyrin repeat containing |
| 27    | 4221         | NM_000244 | MEN1   | Multiple endocrine neoplasia 1 |
| 28    | 4361         | NM_005590 | MRE11A | MRE11 meiotic recombination 11 homolog A (S. cerevisiae) |
| 29    | 4436         | NM_000251 | MSH2   | MutS homolog 2, colon cancer, nonpolyposis type 1 (E. coli) |
| 30    | 4437         | NM_002439 | MSH3   | MutS homolog 3 (E. coli) |
| 31    | 80,198       | NM_025128 | MUS81  | MUS81 endonuclease homolog (S. cerevisiae) |
| 32    | 4609         | NM_002467 | MYC    | V-myc myelocytomatosis viral oncogene homolog (avian) |
| 33    | 4683         | NM_002485 | NBN    | Nibrin |
| 34    | 4691         | NM_005381 | NCL    | Nucleolin |
| 35    | 55,651       | NM_017838 | NHP2   | NHP2 ribonucleoprotein homolog (yeast) |
| 36    | 55,505       | NM_018648 | NOP10  | NOP10 ribonucleoprotein homolog (yeast) |
| 37    | 79,991       | NM_024928 | OBFC1  | Oligonucleotide/oligosaccharide-binding fold containing 1 |
| 38    | 142          | NM_001618 | PARP1  | Poly (ADP-ribose) polymerase 1 |
| 39    | 78,49        | NM_003466 | PAX8   | Paired box 8 |
| 40    | 80,119       | NM_025049 | PIF1   | PIF1 5′-to-3′ DNA helicase homolog (S. cerevisiae) |
| 41    | 54,984       | NM_017884 | PINX1  | PIN2/TERF1 interacting, telomerase inhibitor 1 |
| 42    | 53,47        | NM_005030 | PLK1   | Polo-like kinase 1 |
| 43    | 25,913       | NM_015450 | POT1   | Protection of telomeres 1 homolog (S. pombe) |
| 44    | 54,68        | NM_015869 | PPARG  | Peroxisome proliferator-activated receptor gamma |
| 45    | 55,18        | NM_014225 | PPP2R1A| Protein phosphatase 2, regulatory subunit A, alpha |
| 46    | 55,19        | NM_002716 | PPP2R1B| Protein phosphatase 2, regulatory subunit A, beta |
| 47    | 55,78        | NM_002737 | PRKCA  | Protein kinase C, alpha |
observed in fibroblast has a more significant chromatin condensation or heterochromatin state that contributes to the shorter telomere length.

**Notch signaling cooperate in regulating telomere length in fibroblasts**

Due to the demonstrated cooperativity of TGF-β and Notch signaling in epithelial and fibroblastic cells (Zavadi et al.
we further explored if the observed regulation of telomere length by TGF-β extended to being affected by Notch signaling. Transcriptional regulation of hTERT is believed to play a major role in maintaining chromosome length and stability, limiting telomere degradation, recombination, and end-to-end fusion. We tested the effect of Notch inhibition on hTERT expression in NAF and CAF treated with the gamma-secretase inhibitor, DAPT. Western blotting demonstrated CAF had low hTERT expression as compared to NAF (Fig. 3A). DAPT did not affect TERT expression in NAF or CAF but decidedly increased the elevated SUV39H1 expression in CAF compared to NAF. To investigate the link between SUV39H1 and Notch signaling, dataset from TCGA database was analyzed to examine the correlation between SUV39H1 and Notch downstream genes, HEY1, CCND3 and MYC in clinical PCa specimens by the cBioPortal platform (Fig. 3B, C and Supplementary Fig. 3). The correlation analysis demonstrated that SUV39H1 was positively correlated with HEY1 (Pearson: 0.35, Spearman: 0.35, N = 122), CCND3 (Pearson: 0.47, Spearman: 0.52, N = 122) and MYC (Pearson: 0.21, Spearman: 0.21, N = 122). Notably, cancer epithelia, not stromal fibroblasts, primarily represented the TCGA dataset. Hence, we tested if the apparent correlations were relevant in CAF and context with Notch signaling inhibitions. Treatment with γ-secretase/Notch inhibitor, DAPT, enabled us to test the role of Notch signaling in human prostatic CAF. Telomere length analysis demonstrated CAF treated with DAPT had attenuated telomeres compared to the control group (Fig. 3D). Further telomere-ChIP analysis revealed HP1 DNA recruitment in CAF to be significantly downregulated compared to input (Fig. 3E) in the control group. However, Notch signaling inhibition by DAPT had elevated levels of HP1 DNA recruitment compared to input. Enhanced HP1 recruitment to the shelterin complex can lead to shorter telomeres in CAF, supporting the role of Notch signaling in epigenetic regulation of telomere length.

**Fig. 1** Shorten telomeres in TKO fibroblasts. **A** Flow chart of an overview of the experimental procedures that have been done to measure telomere length. In the first step, isolated mouse fibroblasts were subject to culture. In the second step, genomic DNA was isolated, and the real-time quantitative PCR was performed to measure the Relative Telomere Length (RTL) measurement described by Cawthon (Cawthon 2002). In the Cawthon method RTL was quantified by comparing the amount of the telomere amplification product (T) to that of a single-copy gene (S). **B** Genomic DNA from WT and TKO mouse fibroblasts were analyzed for relative telomere lengths by real-time RT-PCR. The results are plotted as mean RTL. **C, D** Telomerase mRNA expression analysis in WT and TKO mouse fibroblast and human normal and cancer associated fibroblasts by real-time quantitative PCR. The data represents the mean SEM. ***P < 0.001. NAF normal associated fibroblasts, CAF cancer associated fibroblasts.
Considering the telomere length differences in human NAF and CAF, the comparative gene expression analysis on 84 genes (Table 1) related to telomere function was compared. Plotting the detected transcripts on a volcano plot indicated that ten genes were differentially expressed in the CAF from the NAF by twofold or greater (Fig. 4A). Six genes were found to be significantly upregulated, and four were downregulated (Fig. 4A). A group of six telomere-specific proteins (TRF1, TRF2, TPP1, POT1, TIN2, and RAP1), part of the shelterin complex, bind with telomeric DNA to confer telomere protection and length regulation (de Lange 2005). Interestingly, among the six-subunit protein complex genes, TERF1 and POT1 genes were found to be downregulated in CAF, compared to NAF. Next, we determined if the shelterin complex gene expression levels in PCa cells had any bearing on the expression pattern in the prostatic CAF. Analysis of the TCGA interestingly demonstrated only two genes, TERF1 and RAP1, were significantly downregulated in prostate adenocarcinoma tissues compared with non-cancer tissues. In contrast, other shelterin complex genes were not differentially expressed (Fig. 4B, Supplementary Fig. 4).

ARSIs, such as bicalutamide, enzalutamide, and abiraterone are a mainstay in treating recurrent PCa following localized surgical and irradiation therapies. However, the development of ARSI resistance is inevitable. Considering reports supporting the role of fibroblastic TGF-β signaling in castrate resistance (Qi et al. 2013; Kato et al. 2019), we anticipated that telomere length alteration in CAF may contribute to the emergence of castration resistance. Thus, we analyzed the rate of telomere attrition measured at 0-, 10-, and 14-days following treatment with methyltrienolone.
(R1881), a stable androgen and an androgen receptor antagonist, bicalutamide. As expected, fibroblastic telomere shortening was observed over time in all groups. However, the treatment of bicalutamide hastened the rate of telomere attrition compared to control (Fig. 4C). In contrast, R1881 treatment reduced the rate of telomere length decay. The F-ratio value was 9.87274, and the \( P \) value was 0.006912.

**Discussion**

Promoter methylation appears to be an obligitory switch for TGF-\( \beta \)-mediated expression of DNMTs in prostate cancer epithelial and fibroblasts (Banerjee et al. 2014; Zhang et al. 2011). Our results suggested that inhibition of TGF-\( \beta \) signaling via Tgfrb2 promoter methylation and TGF-\( \beta \) antagonist treatment elevated the protein expression of a histone methyltransferase, SUV39H1, in mouse prostatic fibroblasts (Fig. 2). The findings are directly in line with our previous reports, where we found that TGF-\( \beta \) supports DNMT1 protein stability (Banerjee et al. 2014) in prostate fibroblast. Analogously, inhibition of TGF-\( \beta \) signaling led to increased histone methylation, H3K9me3, at telomeric regions along with HP1 (Fig. 2). Telomeres have a high density of DNA repeats that do not contain genes or CpG sequences that are common sites for DNA methylation (Blasco 2007). While DNA methylation might not play a crucial role in telomere length shortening at the telomeric region, the heterochromatic marks such as trimethylation of lysine9 of histone H3 (H3K9me3) and lysine 20 of histone H4 (H4K20me3) may in fact, be more consequential (Schoeftner and Blasco 2009; Janssen et al. 2018; Tardat and Dejardin 2018; Cachione et al. 2019). Overexpression of SUV39H1 enhances H3K9me3 levels at constitutive heterochromatin at telomeric
regions leading to chromatin condensation (Cacchione et al. 2019; Janssen et al. 2018). SUV39H1, histone methyltransferase plays a central role in the establishment of foci enriched for H3K9me3 and HP1, an important epigenetic mechanism for telomere length regulation (Blasco 2007). This model was further supported by the restoration of telomere length when SUV39H1 was silenced (Fig. 2). Interestingly, TGF-β signaling inhibition or knockout led to the elevation of histone methyltransferase, SUV39H1 (Fig. 3). These findings are intriguing since it would suggest CAF, known to have TGFBR2 epigenetic silencing, demonstrate telomeric regulation like that found in embryonic stem-like cells and embryonic fibroblasts (Blasco 2007; Garcia-Cao et al. 2004).

It is an important method chosen to measure telomere length, as the mechanistic findings indicate elevated heterochromatin associated with increased loading of the shelterin complex in condensing the telomeric DNA. We chose to use the reliable method of absolute telomere repeat quantitation. This has the drawback of not revealing physical telomere length but rather the global telomere DNA content, absent the histones. Considering the overwhelming evidence of both reduced telomeric DNA content as well as increased heterochromatin, it would suggest CAF employs both mechanisms of telomere shortening. The epigenetic silencing of the telomerase by TGF-β signaling silencing is supported by the ChIP analysis of H3K9me3 and HP1 loading on the Tert promoter (Fig. 2). However, the upregulation of Notch signaling may support the restoration of telomere length in CAF. In the context of TGFBR2 silencing, the Notch signaling pathway can activate SMAD signaling in the absence of TGF-β, as NICD can bind SMAD3 (Blokhzijl et al. 2003; Luo 2017). Aberrant Notch signaling can be correlated with the development of various diseases, especially tumors, which

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**Fig. 4** Telomere length regulators. A Volcano plot of the telomere real-time RT-PCR array results analyzed by RT² Profiler™ PCR Array of Human Telomeres & Telomerase PCR Array. The black line indicates a 1.0-fold change in gene expression. The pink lines indicate the desired threshold of a 2.0-fold change in gene expression. The blue line indicates the desired 0.05 threshold for the P value of the t test. B Expression levels of shelterin complex genes, TERF1 and RAP1 in PRAD and normal tissues. Gene expression data were compared between PRAD primary tumor and normal control tissues, based on data available in UACLAN database. PRAD, pancreatic adenocarcinoma; TERF1, gene encoding Telomeric Repeat Binding Factor 1; RAP1, Repressor/activator protein 1; TCGA, The Cancer Genome Atlas. C Graph was plotted to access the rate of telomere regression in days’ vs relative telomere length (log value) in CAF treated with Bicalutamide and R1881. CAF: Cancer Associated Fibroblasts. D Proposed model for stromal telomere length regulation: TGFβ and Notch signaling cooperation led to shortest telomere via epigenetic modulation involving recruitment of HP1 which causes heterochromatin state at telomere.
include solid and hematologic malignancies (Gu et al. 2016). In Fig. 3, we revealed that the downregulation of hTERT expression in the CAF was TGF-β dependent, but the Notch signaling seemed to enhance suppressing SUV39H1 expression in CAF. Notch ligand Jagged1 was overexpressed and associated with loss of CpG methylation of H3K4me1-associated enhancer regions (Bhagat et al. 2017). Accordingly, Notch inhibition could increase histone methylation (H3K9me3), providing more docking sites for HP1 binding, thereby increase heterochromatin marks at telomeres, leading to shortening of telomere (Kruk et al. 1995). Thus, with TGF-β signaling inhibition and elevated Notch signaling inherent to CAF, there is a balance in respective telomere length depletion and maintenance that is skewed to shorter due to fewer telomere repeats due to TERT downregulation and DNA condensation from HP1 loading onto the shelterin complex. Telomere shortening is positively correlated with induction of cellular senescence, loss of proliferative capacity (Victorelli and Passos 2017). Although CAF is not senescent, they are described as having a secretory phenotype similar to senescent fibroblasts or those following DNA damage (Coppe et al. 2010). The pro-inflammatory secretory phenotype in fibroblasts, also known as the senescence-associated secretory phenotype (Childs et al. 2015), can promote tumor progression (Demaria et al. 2017).

Telomere shortening is thought to play a prime role in contributing to age-related diseases, such as PCa (Jiang et al. 2008; Song et al. 2010). Here, we proposed a model which describes a novel epigenetic mechanism involving TGF-β signaling in CAF telomere length where shorter telomeres were a product of histone modifications in the hTERT promoter region (Fig. 4D). Age is the most significant risk factor for PCa risk (Stangelberger et al. 2008). Testosterone (T) production by the testis continues to decline while aging, while estradiol (E2) concentration remains constant. Consequently, the ratio between circulating and Intra prostatic E2/T increases (Zhou et al. 2017). Our data suggested that the rate of telomere shortening is increased with androgen targeted therapy compared to the control group. Previous studies showed that AR inhibition using methaneseleninic acid combined with bicalutamide decreased TERT expression (Liu et al. 2010), an enzyme responsible for telomere elongation. In summary, these findings advance our knowledge in stromal telomere length regulation with several limitations which need to be acknowledged while reaching any conclusion. Additional studies are warranted further to investigate the telomerase activity in mouse and human fibroblasts. It will further explore the role of hTERT in controlling heterochromatic telomeres leading to telomere dysfunction and illness.

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**Declarations**

**Conflict of interest** The authors declare that they have no conflict of interest.

**Research involving human participants and/or animals** All the animal experimental procedures of the present study were conducted in accordance with the Institutional Animal Ethics Committee (IAEC) of the Cedars Sinai Medical Center.

**Informed consent** Not applicable.

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