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Telomerase Activity and Myogenesis Ability as an Indicator of Cultured Turkey Satellite Cell Ability for In Vitro Meat Production

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Abstract

Telomerase activity is highly correlated to the proliferation capacity and immortality of cells. To evaluate the possibility of continuous culture, myoblasts were isolated from the Pectoralis thoracicus muscle of newborn turkeys and maintained in 2D (adherence based) and suspension cultures. Furthermore, adherent myoblasts were differentiated into myotubes. Telomerase activity was evaluated in all types of obtained cultures. The expression of telomerase related genes, including TERT1, TERT2, dyskerin, as well as myogenesis related genes, including myogenin, MyoD, MRF1 and MRF5 were measured. Telomerase bands were detected in both adherent and suspended cells, but they were not detected in samples from rat muscle. Myotube differentiation caused a significant reduction in the expression of TERT1, TERT2 and Dyskerin, while MyoD, Myogenin and MRF4 were upregulated in myotubes vs. myoblasts. Long-term culture of suspended myoblasts caused a significant increase in TERT1 levels, with no significant change in expression of myogenesis related genes. Overall, the results show that myoblasts are able to grow in suspension without losing their myogenic properties. Furthermore, upregulation of TERT1 indicates continued proliferation of myoblasts and generation of enough daughter cells necessary for *in vitro* meat production.

Running title: Telomerase activity and myogenic properties of cultured Turkey satellite cells

Keywords: telomerase; myoblasts; myogenesis; 2D cell culture; long-term suspension cell culture; in vitro meat production

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Introduction

Skeletal muscles are generated through proliferation, differentiation and fusion of embryonic myoblasts. In adulthood, muscle growth and regeneration is facilitated by highly proliferative satellite cells, detectable in skeletal muscles of chicken, turkey, pig, cattle and lamb [1]. These cells can be characterized by the expression of markers specific for their lineage, such as ALDH1 and ALDH2 [2]. Satellite cells are located between basal lamina and sarcolemma of the mature muscle fiber, possessing the ability to proliferate and differentiate into myotubes or myofibers [3]. Satellite cell telomere length is a factor of particular significance in muscle regeneration. Telomeres are composed of DNA and multiple protein components. Located at the end of eukaryotic chromosomes, they protect chromosomes end against enzymatic degradation to maintain chromosome stability [4]. They are dynamic structures, with their length able to greatly vary due to environmental conditions [5]. In vertebrates, telomeres consist of repeated sequences of TTAGGG synthesized by the telomerase enzyme [6]. Telomerase activity is present in germ line cells, stem cells, cancer cells, and immortal cell lines, but has not yet been detected in normal somatic cells [7,8]. Its activity can be measured through the analysis of expression of its genes, TERT1 and TERT2, as well as its proven regulators, such as Dyskerin [9]. The absence of telomerase activity in cells allows shortening of telomeres during each cell division, as DNA polymerase is not able to replicate the very ends of the DNA strand [10]. Incompletely replicated ends of chromosomal DNA are inherited by daughter cells and progressively shorten the ends of chromosomes. Through this mechanism, telomerase regulates cell division before senescence [11–13]. Furthermore, telomerase activity seems to be an important factor in production of continuous cell lines. Although the absence of telomerase activity has been demonstrated in somatic adult cells in vertebrates, turkey satellite cells appear to be an exception of that rule [14]. While satellite cells are partly mitotically active in early postnatal skeletal muscles [15], their population shrinks during aging as their proliferation decreases [16], becoming quiescent in mature muscles [17]. However, even such dormant populations of myosatellites have the potential to become mitotically active during muscle repair following injuries [18]. Therefore, their proliferative capacity is partly maintained even during growth and aging. While telomerase activity has not been well studied in myoblasts and satellite cells, it was observed to be relatively high in immortalized mouse C2C12 muscle cell progenitor line [19]. Hence, this study aims at examining the activity of telomerase related genes during an attempted long-term (1 year) suspension culture, as well as compare them to its short-term (3 months) and plastic adherent culture counterparts. The ability to maintain such culture, as well as the maintenance of telomerase related gene expression could suggest that while the muscle satellite cells might lose a significant portion of their stemness due to the *ex vivo* conditions, they can still be successfully propagated for a relatively long periods of time in suspension cultures, which could bear significance for applications such as *in vitro* meat production.

Materials and methods

Animals

5 newborn turkeys (one day old) were killed by cervical dislocation for the purpose of the experiment. Animal experiments were carried out according to the Declaration Helsinki and the Guiding Principles in the Care and Use of Animals (DHEW publication, NIH, 80-23).

Isolation, culture and differentiation of satellite cells

The Pectroralis thoracicus was removed from the turkey using sterile forceps and scissors, mechanically disassociated with sterile forceps and incubated for 30 min in warm (37°C) 0.17% Trypsin+0.085% collagenase solution in Hank's balanced salt, for satellite cell liberation. Subsequently, the tissue was washed twice with turkey plating medium (TPM), which consisted of 89% Dulbecco's Modified Eagle's medium (DMEM), 10% horse serum (Gibco, Grand Island, NY), and 1% penicillin streptomycin fungizone (Gibco). The tissue was resuspended in TPM, triturated through a Pasteur pipette followed by an 18-gauge needle, the cell concentration was estimated using a hemocytometer, and the cells were plated on 0.1% gelatin-coated 100-mm cell culture dishes at a concentration of 300 cells per dish. In the same time, another portion of cells isolated in the same manner was incoculated to an Erlenmeyer flasks containing Mcoy's 5A and 15% Chicken Serum to culture growing cells as suspension under rotation at 110 rpm. In the plastic adherent cultures, after a 24-h attachment period, TPM was replaced with turkey growth medium (TGRM), which consisted of 84% McCoy's 5A, 15% chicken serum (Gibco), and 1% penicillin streptomycin fungizone. Satellite cells were cultured in such manner for the period of for two weeks before growth media were replaced with differentiating media DMEM-4% Horse Serum. After the differentiation process, the samples were collected for further analyses. When it comes to the suspended cultures, the cells were maintained under constant conditions for up to a year, with samples collected after three months.

Telomerase assay

Adherent turkey cells, differentiated cells, suspended turkey cells and rat cells (as negative con-

trol) were subjected to telomerase assay using the TRAPz kit (Millipore, Burlington, MA, USA). To produce cell extract, the cells were dissociated in 200µl CHAPS lysis buffer, which was also added to positive control cells in the kit. Cell extracts were aliquoted and kept in -80°C. For positive control, as well as each negative control samples, a control was prepared by incubating 10µl of the cell extract at 85°C for 30 minutes. The 50 µL reaction mix was prepared by adding 5X TRAPEZE® RT Reaction Mix (5.0 µL), Taq Polymerase about 5 units/µl (0.4 µL), Nuclease Free Water (17.6 µL) and samples: 2.0 µL (Positive extract control, Experimental Samples +/- heat treatment). Tubes were placed in thermocycler to perform PCR cycle. The PCR condition were: 1 cycle for an hour in 60°C, 1 cycle for 2 minutes in 95°C (Taq polymerase was added at this time to the reaction mix), 38 cycles of 95°C for 15 second, 60 °C for 60 second. PCR products were loaded on 10% polyacrylamide gel, with an electrophoresis run at 90 V for 30 minutes. The gel was stained using SYBR green and images were taken using a florescent imaging system.

Evaluation of gene expression using real-time PCR

The expression of genes related to telomerase activity, including TERT1, TERT2 and Dyskerin and myogenesis, including myogenin, myoD, PAX7 and MRF4 was evaluated using real-time PCR [9]. Moreover, the expression of ALDH1 and ALDH2, as muscle progenitor cell markers and Nanog, as marker of self-renewal, were evaluated [2,20,21]. For real-time PCR, total mRNA was extracted from each cell sample using Trizol, in RNase free conditions, dissolved in 20 µl H_2O and stored at -70 °C. RNA concentration was determined using absorbance readings at 260 nm on the Nanodrop-1000 spectrophotometer. cDNA synthesis was performed using a cDNA kit (Sigma-Aldrich, Saint Louis, MO, USA). Primers were designed using the Primer3 software. Primer sequences used in this study are present in Table 1. All qPCR assays used were TaqMan Gene Expression Assays (Thermo Fisher Scientific, Waltham, MA, USA). C_T values for each gene were normalized to the housekeeping Beta-actin gene.

Statistical analysis

ANOVA and Duncan's multiple range test were applied for all statistical analysis using JMP. Before JMP analysis, all real-time PCR results of each gene were normalized using housekeeping gene (*ACTB*). The data were expressed as mean ± SEM. Probability of $p<0.0001$, $p<0.001$ and $p<0.05$ were considered statistically significant.

Ethical approval

The research related to animal use has been complied with all the relevant national regulations and institutional policies for the care and use of animals. The study was approved by Laboratory Animal Care and Use Committee by resolution 19-717-B.

Results

Telomerase detection in myoblasts and myotubes

Telomerase activity was detected in the cultured cells, while it was not present in negative controls including primer-dimer control, rat EDL (extensor digitorum longus) cells and heat-treated controls (**Fig. 1**). In the other gel image (**Fig. 2**), telomerase activity in undifferentiated cells was compared to those subjected to differentiation (fused myoblasts

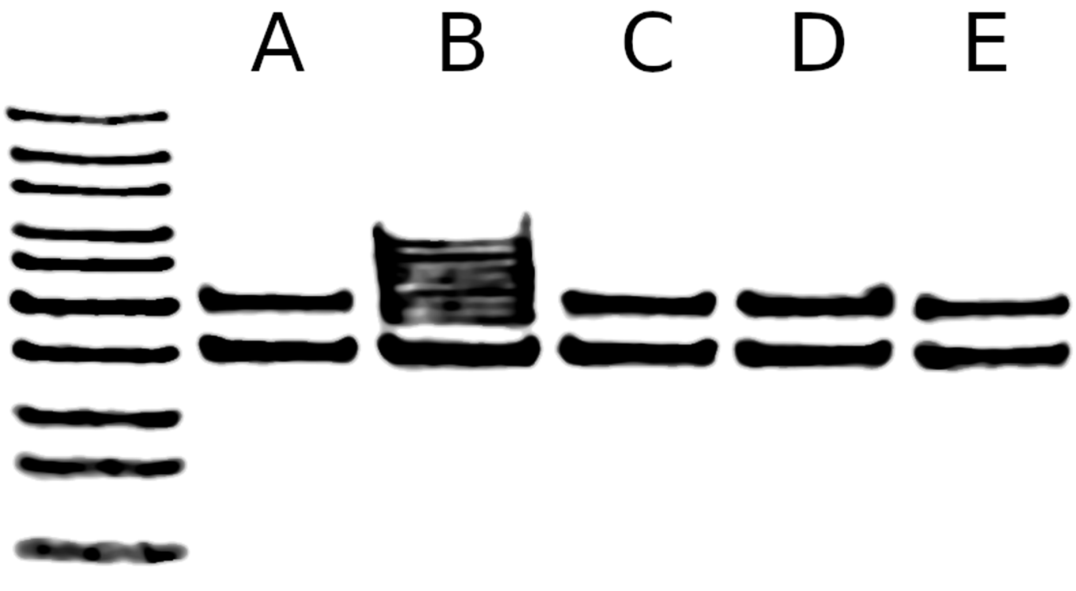


FIGURE 1 Detection of telomerase in the cultured cells. A: Primer dimer control, B: Cultured Turkey cells, C*: Heat treated cultured turkey cells, D: Rat myoblast cells, E*: Heat treated rat myoblast cells. *Samples were treated with 60° C for one hour to inactivate telomerase enzyme. The image is presented as a contrast adjusted negative to ease readability

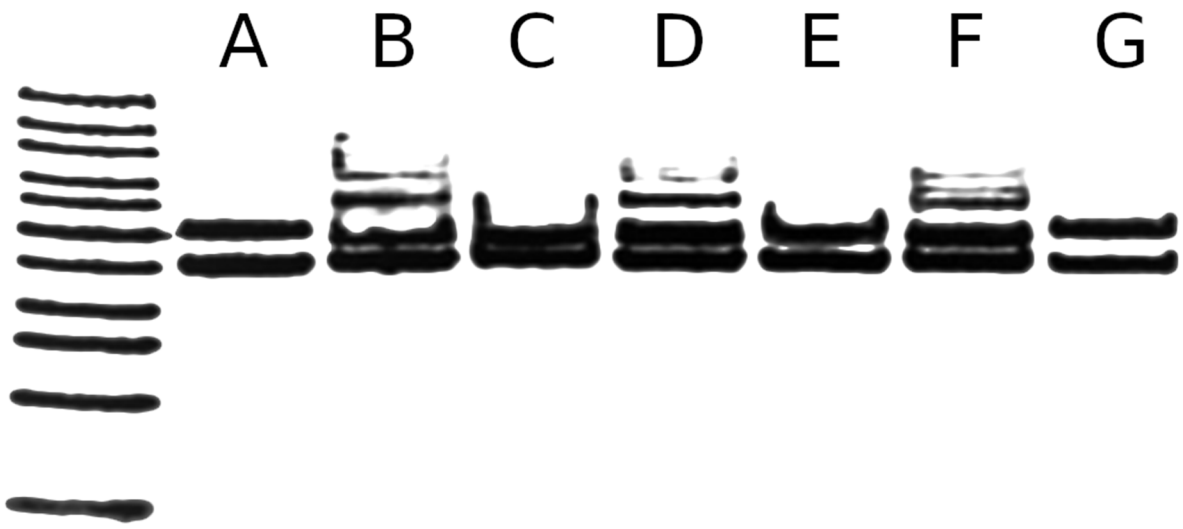


FIGURE 2 Telomerase detection in undifferentiated versus differentiated cells. A: Primer dimer Control, B: kit positive control, C*: Heat treated kit positive control, D:Undifferentiated cells E*: Heat treated undifferentiated cells, F: Differentiated cells, G*: Heat treated differentiated cells. *Samples were treated with 60° C for one hour to inactivate telomerase enzyme. The image is presented as a contrast adjusted negative to ease readability

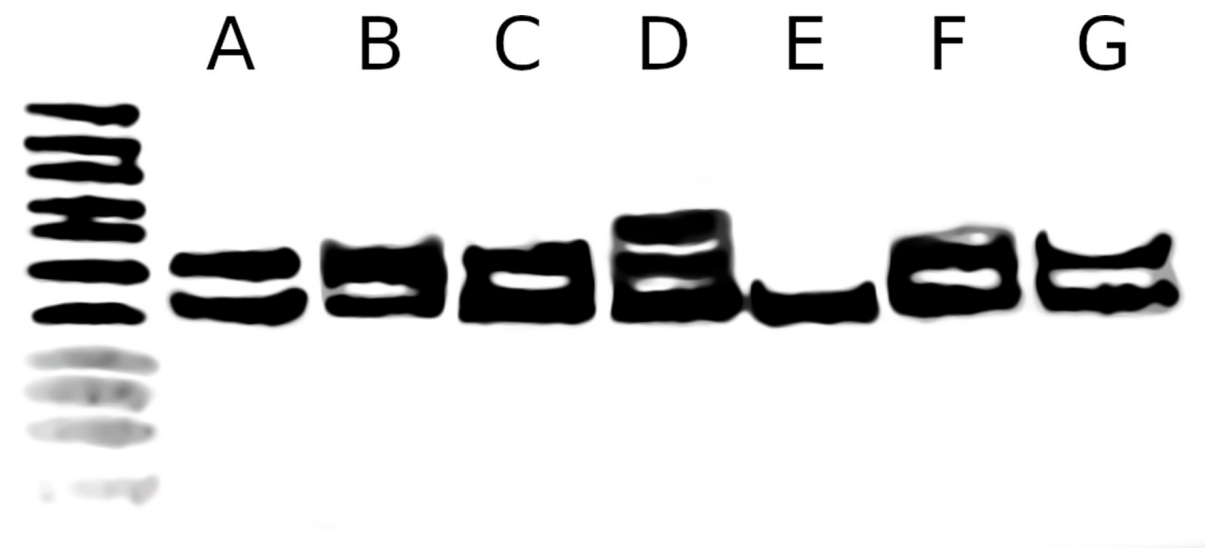


FIGURE 3 Telomerase detection in suspended myoblasts. A:Primer dimer control, B:kit positive control, C: Heat treated kit positive control, D: Short-term turkey cell culture in suspension, E: Heat treated short-term turkey cell culture in suspension, F:Long-term Turkey cell culture in suspension, G: Heat treated Long-term Turkey cell culture in suspension. *Samples were treated with 60° C for one hour to inactivate telomerase enzyme. The image is presented as a contrast adjusted negative to ease readability

or differentiated myoblasts). Telomerase bands were seen in both, corresponding to those seen in the positive control. Furthermore, telomerase activity was evaluated in suspension cultures, as well as compared between short-term and long-term suspension cultures (**Fig. 3**). Telomerase was detected in both short-term and long-term suspension cultures. However, telomerase bands were more similar to kit positive control in long-term cultured cells.

Gene expression

The RT-qPCR results (**Fig. 4**) show upregulation and downregulation of genes including MyoD, MRF4, Myogenin, ALDH1, ALDH2, Nanog, Dyskerin,Tert1, Tert2, and Pax7 after differentiation of the cultured cells. MyoD, MRF4, Myogenin, ALDH1, ALD2 were significantly upregulated compared to Dyskerin, Tert1, Tert2 and Pax7 ($p<0.0001$).

All evaluated genes were upregulated in suspended cells versus adherent cells. However, the upregulation was higher in some genes compared to others. ALDH1 and ALDH2 were highly upregulated, while genes related to myogenesis including MRF4 and myogenin were significantly upregulated compared to Pax7, Myf5 and MyoD ($p<0.0001$). In turn, genes related to telomerase activity including TERT1 and TERT2 were significantly upregulated compared to dyskerin ($p<0.0001$).

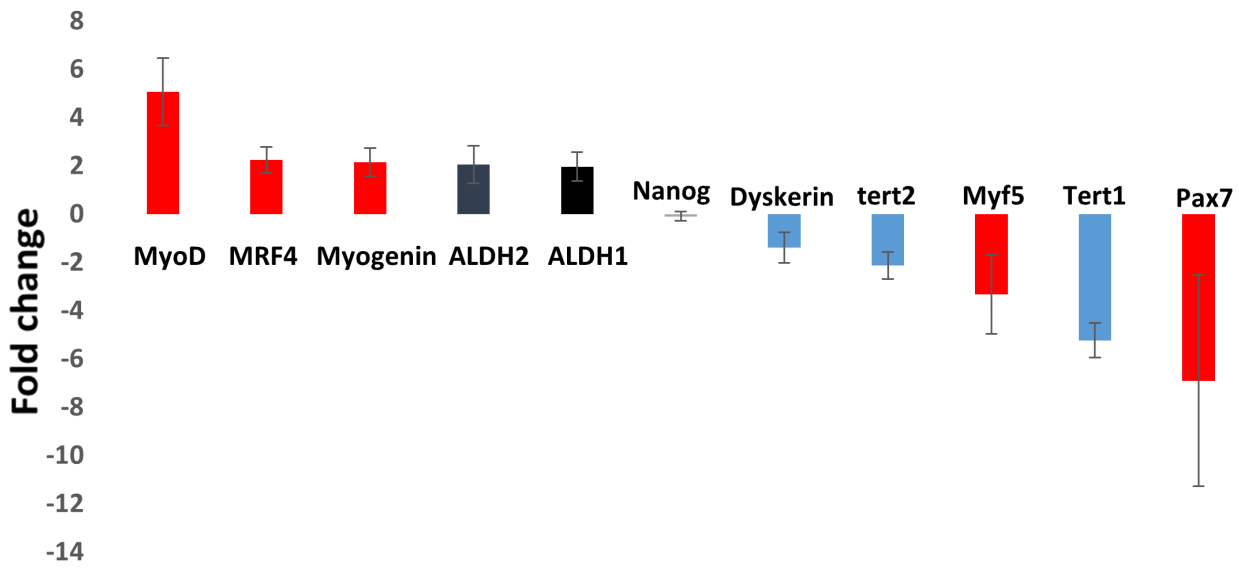


FIGURE 4 Gene expression in differentiated versus undifferentiated cells. The bar chart shows fold changes of genes measured by quantitative RT-PCR. Mean fold changes are expressed on a log2 scale as myotube cell versus myoblast cell values

TABLE 1 Primer sequences were used for the amplification of each gene

Turkey MyoD1	Forward: GCTCCAAGACCTGCCTTCTA
	Reverse: AAACATTGGCCATTTCCCCG
Turkey Myogenin	Forward: ACCGCAACCTCCACTCTCTC
	Reverse: GCCAGCTCAGTTTTGGACCC
Turkey TERT 1	Forward: ACTGCTGAAGACAGTGACGGA
	Reverse: AACCTTCATCGCTTAGTCCAGT
Turkey TERT 2	Forward: GCTGGCAAGTCTCTGTGACTCTT
	Reverse: GGAGAGGGAGCTGCTCAGAAC
Turkey MYF5	Forward: AGCTGCCAGTTCTCCCCATC
	Reverse: GTGCTCCTCTTCCTCAGGGC
Turkey MRF4	Forward: TCCGACTTCCTGAGCACCTG
	Reverse: TTTCTCCACCGCCTCTTCCG
Turkey Pax7	Forward: TGGCGGATGTGGTTGGGTAA
	Reverse: TAGAAGTGTCCACCCCGCTG
Turkey β-actin	Forward: TACTCCCACAGCCAGCCATG
	Reverse: TGGGGTACTTCAGGGTCAGGA
Turkey Dyskerin	Forward: GTTAGCGACTACCCCCGACC
	Reverse: GTGGAGACCCCGTTCCTACG
Turkey ALDH1	Forward: AATCGCTCCCTTGCTGTTGA
	Reverse: AATCGCTCCCTTGCTGTTGA
Turkey ALDH2	Forward: GCTAGTCGTAAAGACCCCCG
	Reverse: TCTGGCACGGAAAATGCTCA
Turkey Nanog	Forward: CAGAGCAGAAAACGCCCAGC
	Reverse: CCTTGTCCCACTCTCACCTTTA
Turkey GAPDH	Forward: TACTCCCACAGCCAGCCATG
	Reverse: ACGGCAGGTCAGGTCAACAA

Finally, when it comes to expression in suspension cultured cells long-term versus short-term, the results are presented in **figure 6**. The expression of telomerase related gene TERT1 was significantly upregulated compared to TERT2 and Dyskerin ($p<0.0001$). However, there were no significant differences in the expression of myogenic-related genes.

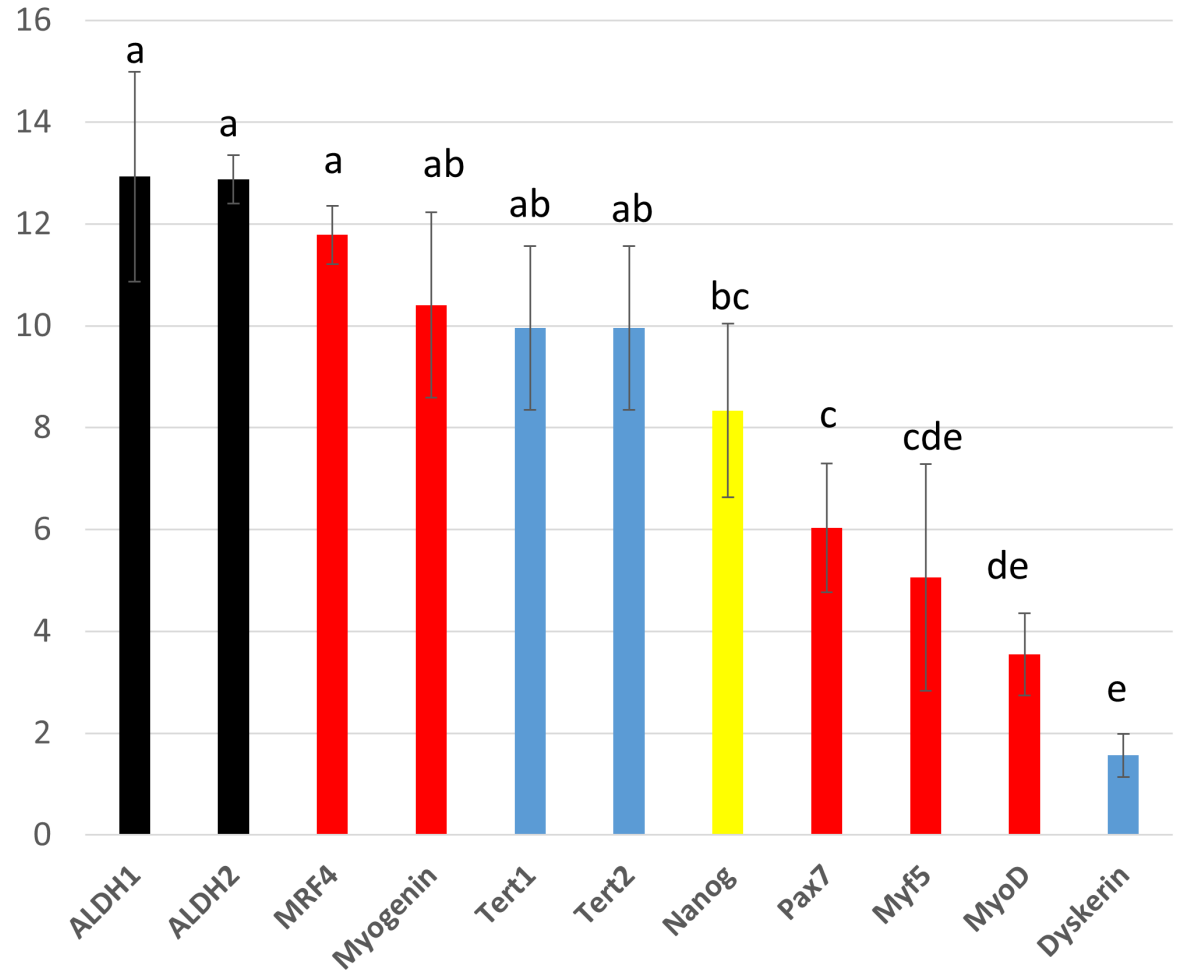


FIGURE 5 Gene expression in long-term suspension culture versus adherent cells. The bar chart shows fold changes of genes measured by quantitative RT-PCR. Mean fold changes are expressed on a log2 scale as long-term suspended culture versus adherent cells FC values

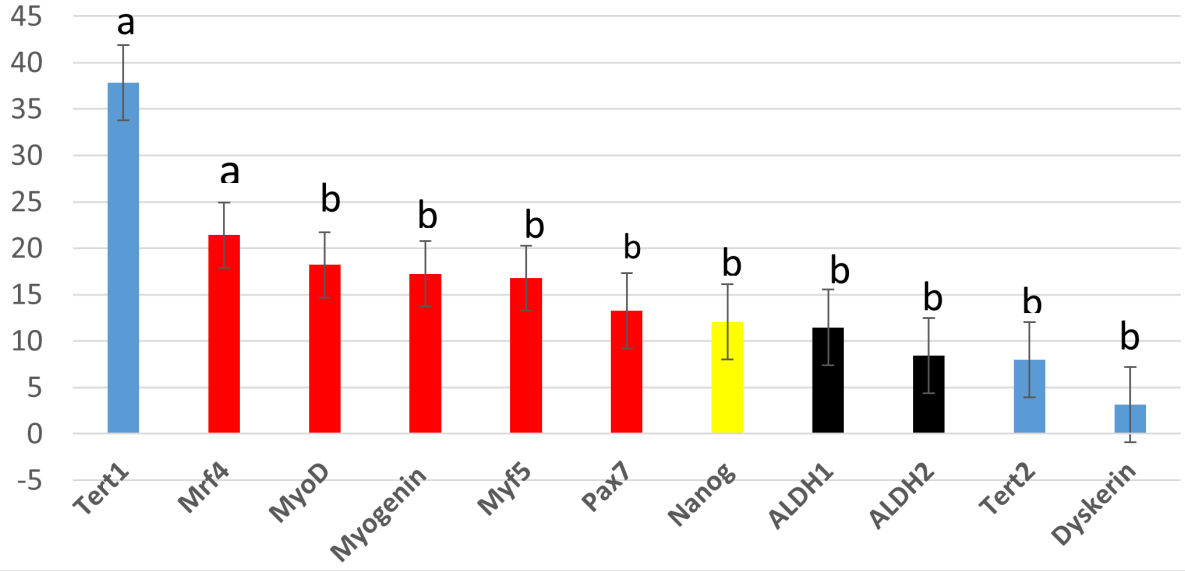


FIGURE 6 Gene expression in short-term (3 month) versus long-term (12 month) cell culture in suspension. The bar chart shows fold changes of genes measured by quantitative RT-PCR. Mean fold changes are expressed on a log2 scale as short vs long-term suspended culture cell values

Discussion

Telomerase activity is a very important factor in continuous cell line production, as it prevents cell senescence and death. While most somatic cells do not exhibit telomerase activity, it has been reported in some tissues of certain animals. Satellite cells are muscle stem cells which show robust telomerase activity throughout adult life. However, their immediate primary myoblast progeny dramatically downregulates telomerase activity [3]. Despite that, it has been previously shown that turkey breast muscle satellite cells exhibit a certain extent of telomerase activity [14]. In this research, satellite cells were isolated and cultured as adherent primary myoblasts and suspended primary myoblasts to evaluate their ability to preserve their telomerase properties after a long-term culture. Results of this research indicated that there is telomerase activity in cultured turkey myoblasts, as telomerase bands were detected in those cells while rat myoblasts didn't show any bands (**Fig. 1**). Furthermore, differentiation of myoblasts into myotubes was performed and telomerase activity was observed in myotubes (**Fig. 2**), which may be due to the presence of a population of non-differentiated cells, or remains of the previous high telomerase activity that could disappear after more passages. In the mature muscle fibers, there are quiescent satellite cells located in sarcolemma which can be induced to proliferate after injury [17]. Telomerase detection in differentiated myotubes is probably caused by the presence of quiescent satellite cells.

Furthermore, suspension cultures are being used increasingly in the biotechnology as they have the ability to be easily scaled up to produce therapeutic proteins or edible biomass [22]. In this research, to scale up myoblast cell culture, myoblasts were transferred placed in a suspension culture flasks and were cultured for a long-term (a year). After the culture period, telomerase activity was detected in the cells. As **figure 3** shows, the telomerase bands were more similar to positive control after long-term suspension culture. Therefore, culture of myoblast cells in suspension had no adverse effect on telomerase activity, with long-term culture probably inducing more telomerase activity. As can be seen in Figure 4, the expression of genes related to telomerase activity and myogenesis in myotubes versus myoblast is indicating down regulation of telomerase related genes including TERT1, TERT2 and Dyskerin. It has been demonstrated that in somatic cells the expression level of TERT decreases after differentiation [12], with overexpression of this gene able to cause significant raise in telomerase activity [23]. Dyskerin is a nuclear protein that binds to the H/ACA box of telomerase RNA and forms ribonucleoprotein (RNP), with its dysfunction causing telomerase shortening [24]. In turn, **figure 5** shows TERT1, TERT2, and Dyskerin expression in suspended cells compared to adherent cells. While upregulation of all those genes is seen, TERT1 and TERT2 expression was significantly higher than Dyskerin. In a research on immortalized human mammary epithelial cells, despite increased Dyskerin, overexpression of hTERT was observed. However, there was no correlation between hTERT expression or telomerase activity and Dyskerin expression level., A significant upregulation of TETR1 was detected compared to other genes in cells that has been cultured in suspension for long-term, compared to short-term (**Fig. 6**) indicating that long-term culture of myoblasts in suspension probably induces higher expression of TERT1 and telomerase activity.

Expression level of myogenic genes in myotubes has been different than in myoblasts. As it is shown in **figure 4**, MyoD, MRF4 and Myogenin were upregulated and Myf5 and PAX7 were downregulated in myotubes compared to myoblasts. Myogenic regulatory factors including Myf5, myogenin and MyoD are members of the helix-loop-helix family of transcription factors that control differentiation of skeletal muscle cells during prenatal and postnatal development [25]. As differentiation of myoblasts into myotubes occurs, the MyoD locus relocates to the lumen of the nucleus where the factors that promote MyoD expression are located [26]. In the next step, MyoD induces myogenic expression, which causes downregulation of Myf5 [27]. Paired box7 (Pax7) is one of the important factors in the regulation of differentiation and specification [28]. It has been shown that Pax7 inhibits differentiation of myoblasts by inhibiting Myod, resulting in myogenin activation [29,30].

All of the investigated genes related to myogenesis has been upregulated in suspended versus adherent cells. MyoD, Myf5 and Pax7 upregulation was significantly lower than that of Mrf4 and myogenin. Higher expression of myogenin probably indicates that the presence of MyoD in cells increases myogenin expression. While significant upregulation of myogenin indicates differentiation of myoblasts, significant upregulation of TERT1 in long-term versus short-term cultured myoblasts indicates high proliferation capacity of the myoblast cells in suspension. The data indicates that there should be both undifferentiated and differentiated myoblasts present in the suspension cell culture. It has been shown that ALDH1 and ALDH2 expression is a good marker of human myoblasts cells [31]. Presence of ALDH1 and ALDH2 in differentiated and undifferentiated cells indicates the presence of myoblasts cells in all culture systems that we were evaluated.

Conclusions

To our knowledge, this the first report on the establishment of a year-long myoblast cell culture in suspension, with the results indicating that such form of culture is possible and viable. However, im-

provement of cell culture conditions, such as the addition of growth factors to cell culture media to prevent differentiation of myoblasts, may help to obtain a better efficiency of large-scale *in vitro* cell production. *In vitro* meat manufacturing is a prospective and widely investigated potential source of human nutrition [32]. Hence, as the establishment of suspended muscle cell lines is a promising method for successful culture of those cells in large scale for meat production purposes, it could become a significant contributor to the development of this novel industry.

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Conflict of interest

The authors declare they have no conflict of interest.

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