Muscle Stem Cells in Children with Cerebral Palsy have Shorter Telomeres and Differential Metabolic Profiles Reflective of Premature Senescence

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INTRODUCTION: Children with cerebral palsy (CP) develop muscle contractures during postnatal muscle growth. Contractured CP muscles are generally shorter and smaller and contain myofibers of reduced diameter and overstretched sarcomeres[1,2], compared to typically developing (TD) muscle. We have previously shown that the resident muscle stem cell pool, i.e. satellite cells (SCs), are significantly reduced in contractured hamstring muscle tissue of children with CP[3]. We subsequently demonstrated that SCs isolated from these muscles were also impaired in their capacity to generate muscle fibers *in vitro*[4]. The loss of myogenic potential in these SCs was due to epigenetic imprint of DNA hypermethylation that led to genomic changes favoring premature stem cell expansion into myoblast progenitors over self-renewal and differentiation. This was validated by the restoration of muscle-growing potential of these SCs by treating them with DNA hypomethylating agents such as FDA-approved Azacitidine (AZA)[4]. Cellular senescence is a terminal state of loss of self-renewal capacity after multiple cell divisions. It is linked to telomeres shortening, genomic instability, metabolic changes, mitochondrial dysfunction and oxidative stress[5]. Here we tested the hypothesis that DNA hypermethylation activated genetic programs promoting cell division and proliferation leading to premature depletion of muscle SC pools through mechanisms of cellular senescence.

METHODS: IRBs from Rady Children Hospital of San Diego, CA and Ann & Robert H. Lurie Children's Hospital of Chicago, IL provided ethical approval, and appropriate assents/consents were obtained from children and their parents. SCs (defined as CD31-/CD45-/CD56+ cells) were isolated from hamstring and vastus lateralis muscle from TD and spastic CP children. SCs were expanded into proliferating progenitor myoblasts cultures in 0.5% gelatin-coated plastic dishes, using a 20% FBS medium. Myoblast treatment with AZA consisted of exposing cells to 5 uM of AZA for 24 hours. Replicative cellular senescence was induced by continual passaging of myoblasts in culture. RNA-sequencing followed by clustering analysis on Cytoscape were used to characterize changes in gene expression of CP *vs*. TD myoblast cultures at low passage number (P4). ACEA's xCELLigence RTCA was used to measure proliferation rate (doubling time) of CP *vs*. TD myoblasts at high (P12) *vs*. low (P4) passages. Relative Telomere Length (RTL) was measured in myoblasts at P12 *vs*. P4 using Flow cytometry-based fluorescent *in situ* hybridization (Flow-FISH). Targeted hydrophilic metabolomics using LC-MS was performed to assess metabolite profiles at low passages. Agilent Seahorse XFp Real-Time ATP Rate Assay was used to assess mitochondrial respiration (OCR) and glycolytic capacity (ECAR) of myoblasts at baseline, following oligomycin (inhibitor of Complex V) and rotenone+antimycin A (inhibitors of Complex I+III) at low and high passages. Data were compared by 2-way ANOVA and individual groups by multiple t-tests for n=4-7 per group, as detailed in Figure 1. Significance level was set to $p<0.05$.

RESULTS: We demonstrate that continual passaging (from P4 to P12) increased doubling time (i.e., decreased proliferation rates) in both CP and TD myoblasts (Fig. 1A). With cell passaging, there was a decrease in telomere length in TD myoblasts but CP myoblasts had shorter telomere lengths from low passages that did not change with passaging (Fig. 1B). Metabolite profiles of CP and TD were distinctly classified into two principal components using partial least squared-discriminant analysis. Functionally, mitochondrial respiration (OCR) (Fig. 1C) and glycolysis (ECAR) (Fig. 1D) were both significantly increased at low passages in CP myoblasts, but not different at high passages. Our gene clustering analysis identify upregulation of at least two important signaling pathways in proliferating CP myoblasts, namely the EGFR – PI3K – AKT and the IL6ST (gp130) – JAK – STAT axes. These pathways upregulated multiple downstream genes such as HIF-1a, CREB, BMI1 and ATM that control cell division, DNA damage and repair, reactive oxygen species (ROS) turnover, metabolism, telomere length and cellular senescence. These pathways were normalized in expression after treating CP myoblasts with 5uM AZA.

DISCUSSION: Telomeres are nucleoprotein structures that protect the ends of chromosomes from DNA degradation. Most adult cells, including stem cells, progressively lose telomeres with increased cell division and tissue renewal, contributing to genomic instability, ageing, disease and cellular senescence [6]. Our data show that myoblasts in children with CP might have premature senescence. Senescent cells have been shown to experience dramatic changes in terms of gene expression, metabolism, epigenome and contribute to overall muscle tissue damage [6]. Our data strengthen the notion that DNA hypermethylation in CP myoblasts is associated with activation of genetic programs promoting premature depletion of muscle SC pools through mechanisms of cellular senescence.

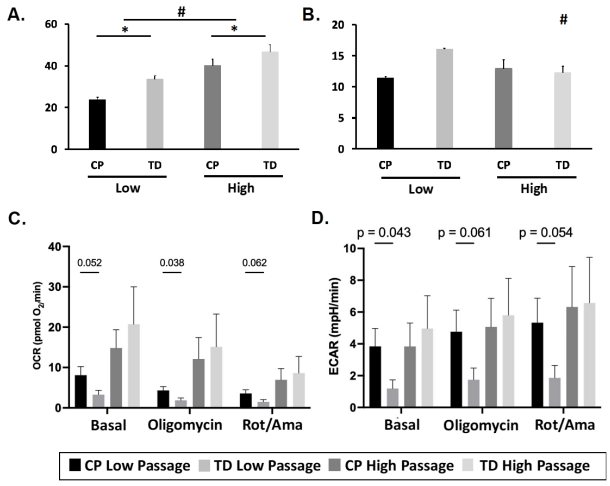

Figure 1: (A) Cell culture doubling time for CP and TD myoblasts, at low vs high passages (in hours). Group (*) and Passage (#) effects by 2-way ANOVA ($p<0.05$, n=6-7); **(B) Relative Telomere Length (RTL)** for the same Groups and Passages. Group x Passage interaction effect by 2-way ANOVA (#$p<0.05$, n= 4-7); **(C) Oxidative Capacity Rate (OCR) (D) Extracellular Acidification Rate (ECAR)** (for the same Group and Passages, at baseline and after oligomycin and rotenone+antimycin A (Rot/Ama) exposure. P-values for t-tests (n= 6-7).

SIGNIFICANCE/CLINICAL RELEVANCE: Since SCs are critical for postnatal muscle growth, their reduction in number and function in contractured CP muscle could represent a mechanistic explanation for the lack of radial and longitudinal growth observed in these children. Our results have important implications for therapy since loss of SCs and homeostasis, possibly due to premature cellular senescence, suggest that it is almost impossible for these muscles to grow physiologically without introduction of new therapeutic modalities targeting SC self-renewal and differentiation. A multimodal nonsurgical treatment combining physical therapy with biological enabling using hypomethylating agents could be a solution.

REFERENCES: [1]Smith et al. 2011, J Physiol 589:2625; [2]Barber *et al.*, 2011, Dev Med Child Neurol 53:543; [3]Dayanidhi *et al.* 2015, J Orthop Res, 33:1039; [4]Sibley *et al.* 2021, FASEB J, 35:e21928; [5]Di Micco *et al.* 2021, Nat Rev Mol Cell Biol 22:75-95; [6]Petrocelli *et al.* 2023, Aging Cell, e13936.

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