Patient-derived cell culture model to investigate osteogenesis in Treacher Collins syndrome

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INTRODUCTION: Nearly one-third of all congenital birth defects are associated with craniofacial malformations, which usually arise due to disturbances that occur during embryonic development, specifically in neural crest cells (NCCs) development. Treacher Collins syndrome (TCS) is a rare congenital craniofacial disorder, mainly characterized by hypoplasia of the mandible and zygomatic arches, cleft palate, and malformations in the internal and middle ear. Currently, there are no effective treatments for the syndrome, only invasive reconstructive surgeries (e.g., mandible distraction) that are rarely 100% reconstructive, especially for the severely affected cases. Most cases (~93%) are caused by loss-of-function mutations in *TCOF1*, that impair ribosome biogenesis and rDNA damage response, which results in apoptosis of NCCs progenitors and compromises the formation of craniofacial bones and cartilage. A lot of TCS early embryogenesis has been uncovered, but little is known about the later stages, such as in osteogenesis. Redox state maintenance and ribosome biogenesis, both altered in TCS, are crucial for adequate osteogenesis. Therefore, it is relevant to investigate TCS osteogenesis. Our preliminary data in 2D culture models indicate that TCS osteogenesis is impaired. To further investigate and understand which cellular and molecular mechanisms are altered, here we propose to evaluate TCS osteogenesis using a system that mimics the *in vivo* biology with greater fidelity, 3D culture with patient-derived cells (neural crest cell-derived mesenchymal stem cells, nMSCs) (**Fig. 1A**). Our main hypothesis is that TCS osteogenesis is impaired by alterations in cell proliferation and/or differentiation (**Fig. 1B**).

METHODS: *Monolayer osteogenic differentiation and characterization:* nMSCs were seeded in a cell density of 2,500 cells/cm^2 and osteogenic differentiation was carried for 21 days. Samples undergoing osteogenic differentiation (in osteogenic media – OM) were collected on days 7, 14 and 21. Samples in control media (basal media without osteogenic factors – CTRL Media) were collected on day 21. Characterization was done with Alizarin Red Staining. *Spheroid formation:* nMSC-spheroids were generated with the hanging drop method (4,000 cells/spheroids) (2). *Spheroid encapsulation, cultivation, and collection:* after generation, nMSC-spheroids were suspended in fibrin gel (20 spheroids/gel) and seeded on PDMS (Polydimethylsiloxane) molds that were placed on top of petri dishes coated with agarose 2%. Spheroids were also cultivated in OM or CTRL Media, and were collected on days 0, 14 and 21 of the osteogenic differentiation, and the ones in CTRL Media, on day 21. *Characterization of osteogenic differentiation - spheroids:* DNA quantification and Calcium content assays (N=4-6, for each group and time point). *Statistical analysis:* One-way ANOVA with Bonferroni's post-hoc test (GraphPad Prism 8 software). *Ongoing assays:* RT-qPCR for osteogenic markers (*BGLAP*, *Osteopontin* and *RUNX2*).

RESULTS: hiPSCs (human induced pluripotent stem cells) from 3 healthy individuals (HI), 2 clinically different TCS patients with different variants in *TCOF1* ('diseased individuals', DI), and 3 *TCOF1*-knockout lines (CRISPR *TCOF1*-KO) were first differentiated into iNCCs (neural crest cells-derived from hiPSCs), then to nMSCs (**Fig. 1C**) (1). Characterization of nMSC monolayer osteogenic differentiation showed no staining for Alizarin Red in DI cells, whereas HI cells were strongly stained (**Fig. 1D**). nMSC-spheroids were efficiently and consistently generated (**Fig. 2A-B**). No differences in DNA content were observed between samples (**Fig. 3A**). There was a significant increase in Calcium content in all samples on OM day 21, especially for cells *TCOF1*-deficient (DI 2 and CRISPR HM delC) (**Fig. 3B**). A similar trend is observed for Calcium/DNA data, showing that *TCOF1*-deficient cells (DI 2 and CRISPR HM delC) have the highest amount of calcium mineralization per cells on OM day 21 (**Fig. 3C**).

DISCUSSION: Establishing a 3D culture model with TCS patient-derived nMSCs is of great importance to understand and investigate processes such as osteogenesis with greater fidelity. Our preliminary data, both on 2D and 3D culture systems, suggests that osteogenesis is altered in DI cells. Cell density and cell-to-cell interaction seem to play an important role in TCS osteogenesis, as cell culture in low density led to impaired osteogenesis (2D culture data), whereas higher cell density resulted in accelerated osteogenesis (3D culture data) in DI cells. Further analyses, such as gene expression assays (RT-qPCR), are necessary and are being evaluated to better understand how these processes may be involved in TCS altered osteogenesis.

SIGNIFICANCE/CLINICAL RELEVANCE: Around one-third of all congenital defects are associated with craniofacial malformations and currently, there are no efficient treatments for these malformations, including for TCS. Therefore, uncovering TCS osteogenesis may not only contribute to the understanding of other craniofacial syndromes, but it can also open venues for the development of prospective treatments to improve patient's bone regeneration and diminish the invasiveness of reconstructive surgeries.

REFERENCES: (1) Kobayashi et al., Stem Cell Reports. 2020 Sep 8;15(3):776-788; (2) Hutton et al., Tissue Eng Part A. 2013 Sep;19(17-18):2076-86.

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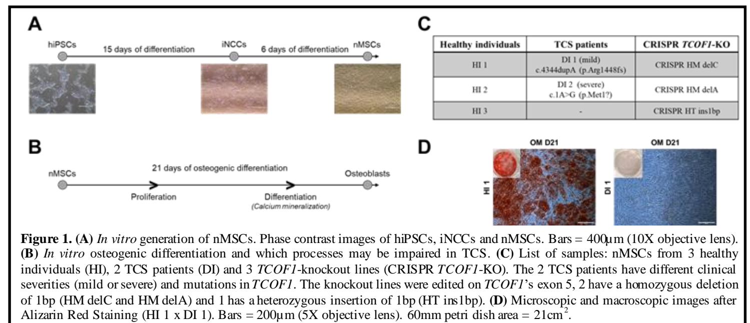

Healthy individuals	TCS patients	CRISPR *TCOF1*-KO
HI 1	DI 1 (mild) c.4344dupA (p.Arg1448fs)	CRISPR HM delC
HI 2	DI 2 (severe) c.1A>G (p.Met1?)	CRISPR HM delA
HI 3	-	CRISPR HT ins1bp

Figure 1. **(A)** *In vitro* generation of nMSCs. Phase contrast images of hiPSCs, iNCCs and nMSCs. Bars = 400µm (10X objective lens). **(B)** *In vitro* osteogenic differentiation and which processes may be impaired in TCS. **(C)** List of samples: nMSCs from 3 healthy individuals (HI), 2 TCS patients (DI) and 3 *TCOF1*-knockout lines (CRISPR *TCOF1*-KO). The 2 TCS patients have different clinical severities (mild or severe) and mutations in *TCOF1*. The knockout lines were edited on *TCOF1*'s exon 5, 2 have a homozygous deletion of 1bp (HM delC and HM delA) and 1 has a heterozygous insertion of 1bp (HT ins1bp). **(D)** Microscopic and macroscopic images after Alizarin Red Staining (HI 1 x DI 1). Bars = 200µm (5X objective lens). 60mm petri dish area = 21cm^2.

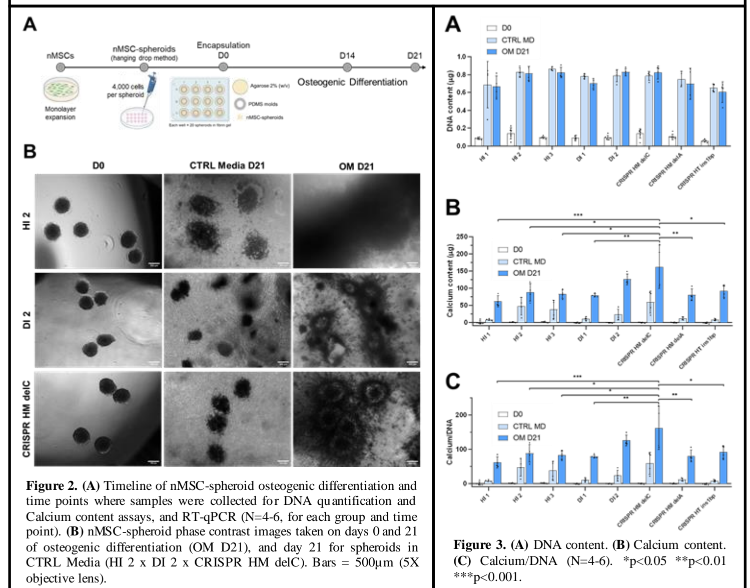

Figure 2. **(A)** Timeline of nMSC-spheroid osteogenic differentiation and time points where samples were collected for DNA quantification and Calcium content assays, and RT-qPCR (N=4-6, for each group and time point). **(B)** nMSC-spheroid phase contrast images taken on days 0 and 21 of osteogenic differentiation (OM D21), and day 21 for spheroids in CTRL Media (HI 2 x DI 2 x CRISPR HM delC). Bars = 500µm (5X objective lens).

Figure 3. **(A)** DNA content. **(B)** Calcium content. **(C)** Calcium/DNA (N=4-6). *$p<0.05$ **$p<0.01$ ***$p<0.001$.