Multipotential Differentiation of Cells Derived from Trinity Evolution®

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INTRODUCTION

The multipotentiality of adult mesenchymal stem cells (MSCs) is defined by the capacity of these cells to undergo extensive cell expansion and differentiate into multiple lineages. Standard criteria used to identify MSCs include plastic-adherency, expression of specific cell surface markers, and the ability of the cells to differentiate and form connective tissues such as bone, fat, and cartilage under different conditions[1,2]. In this study, samples of Trinity Evolution tissue that had been stored for 1 month in vapor phase liquid nitrogen were thawed, explanted, and evaluated to determine if MSCs were present in the cultures. Following cell migration out of the tissue, the cells were expanded in culture to enable *in vitro* testing. Subsequently, differentiation assays were performed to assess the osteogenic and adipogenic capcity of the explanted cells.

PROLIFERATION & MORPHOGENESIS

Samples of Trinity Evolution were thawed as per the package insert and plated onto tissue culture dishes. After a brief incubation period, the tissue was enzymatically treated[3]. The treated tissue was then transferred into tissue culture flasks containing culture medium. Subsequently, the medium was exchanged every third or fourth day to allow for cell expansion. During the culture period, the flasks were examined for cell adherence and cell growth. Cells were observed migrating from the chips to the tissue culture flask surface and cell proliferation occurred until confluency was reached. Cell phenotype was distinctly spindled-shaped and characteristic of MSCs. *Figure 1* illustrates typical elongated cell morphology of explanted cells from Trinity Evolution tissue (images were taken at 10x magnification).

Cell Morphology

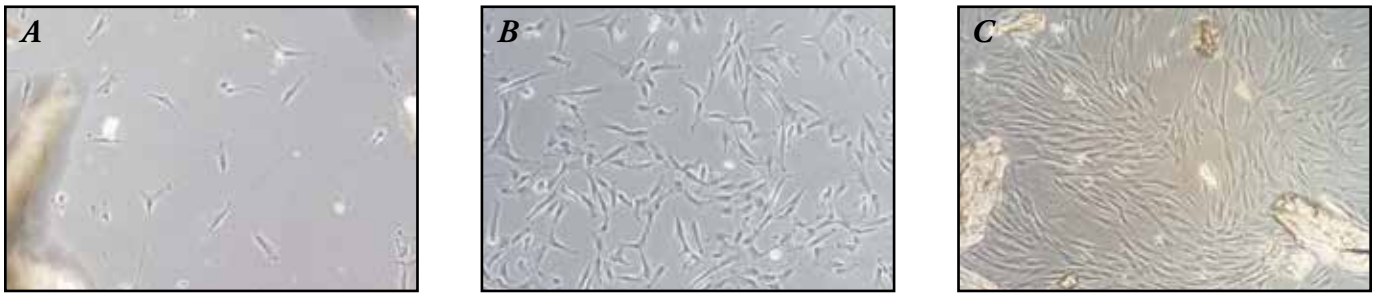
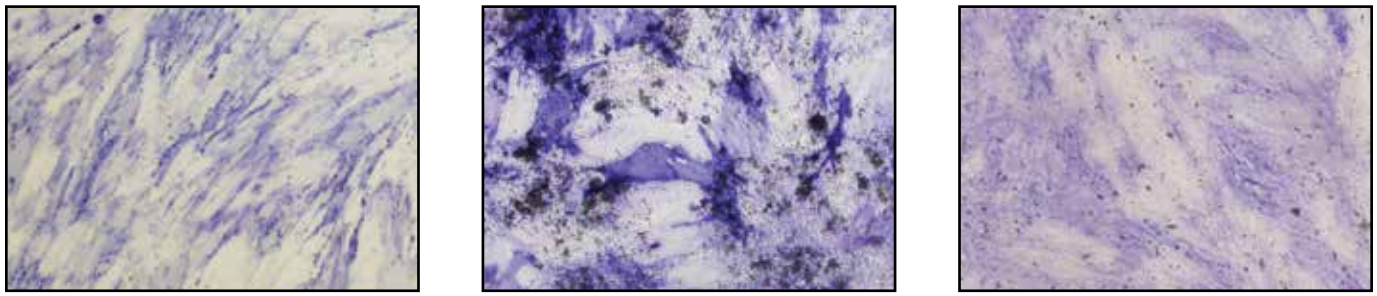


Figure 1: Explanted cells from Trinity Evolution tissue exhibit an elongated spindle-shape typical of MSCs during culture: (A) cells growing out of bone chips, (B) proliferating cells, and (C) cells having reached confluency.

OSTEOGENESIS

To assess the capability of explanted cells from Trinity Evolution tissue to differentiate into the osteogenic lineage, cells from passage 1 (P1) expansion were trypsin-treated from confl uency and re-seeded into 24-well tissue culture plates. The cells were induced with osteogenic stimulatory medium4 containing dexamethasone and ascorbic acid, with the addition of ß-glycerophosphate once cell multilayering was observed. Media was exchanged every third or fourth day, and the cell cultures were osteogenically induced for up to 8 weeks. Alkaline phosphate (ALP) activity (an early osteogenic marker) and initiation of mineralization (calcium deposition) of the cells were qualitatively assessed at 2, 4 and 8 weeks. ALP activity was determined by using a histochemical method (Sigma) that provides a visual localization of ALP activity by the deposition of a blue salt[5]. *Figure 2* illustrates the positive staining of ALP as early as 2 weeks with greater staining intensity at 4 weeks and an eventual decrease by 8 weeks (images taken at 10x magnification). This pattern coincides with increasing mineralization that is induced as ALP expression decreases.

ALP Staining

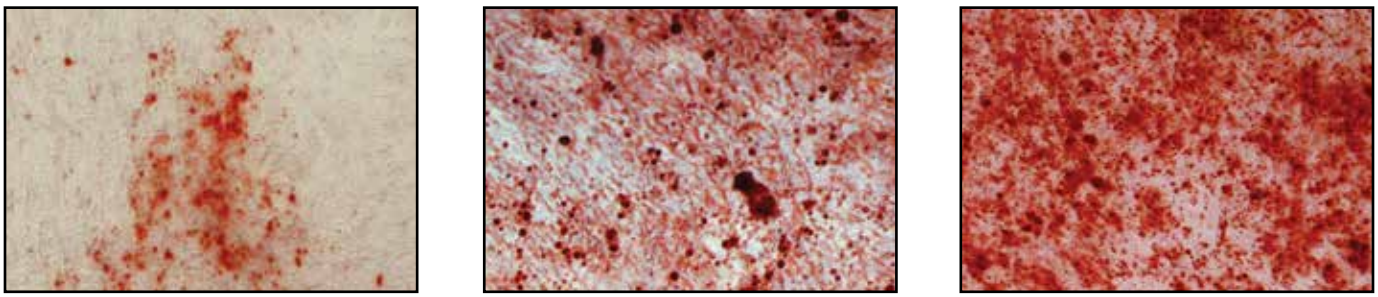


2 weeks ***4 weeks*** ***8 weeks***

Figure 2: Positive ALP staining (blue stain) observed in explanted cells from Trinity Evolution tissue at 2, 4 and 8 weeks in culture.

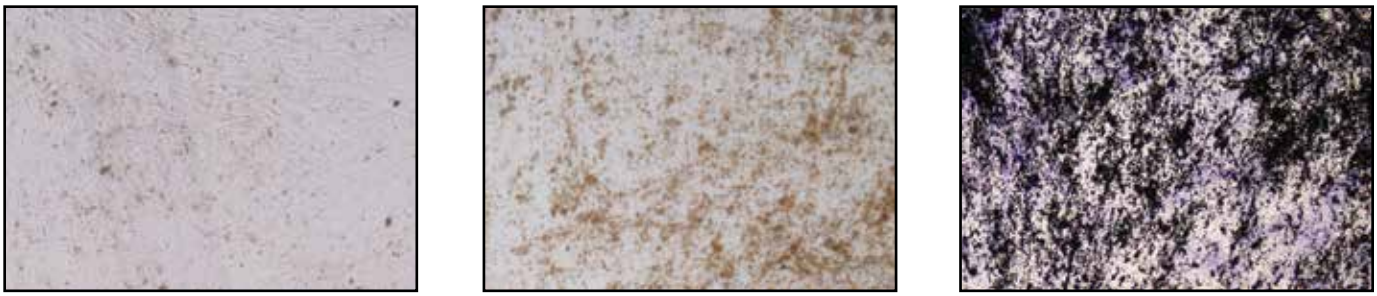
Mineralization of the cell monolayer was detected by Alizarin Red S and von Kossa stain[2,6]. Alizarin Red S forms a complex with calcium, showing a positive red stain for mineralizing nodules in the culture. The von Kossa staining is a precipitation reaction in which silver ions replace calcium and form a black deposit in the presence of calcium phosphate. Both of these staining techniques demonstrate positive scores for osteogenic differentiation and mineral deposition in the extracellular matrix of the explanted cell cultures as shown in *Figure 3*. The results revealed distinctly increasing intensity of staining, which is associated with increasing mineralization over time (images taken at 10x magnification). These findings demonstrate the ability of the explanted cells to differentiate into the osteogenic lineage.

Alizarin Red S Staining



2 weeks ***4 weeks*** ***8 weeks***

von Kossa Staining



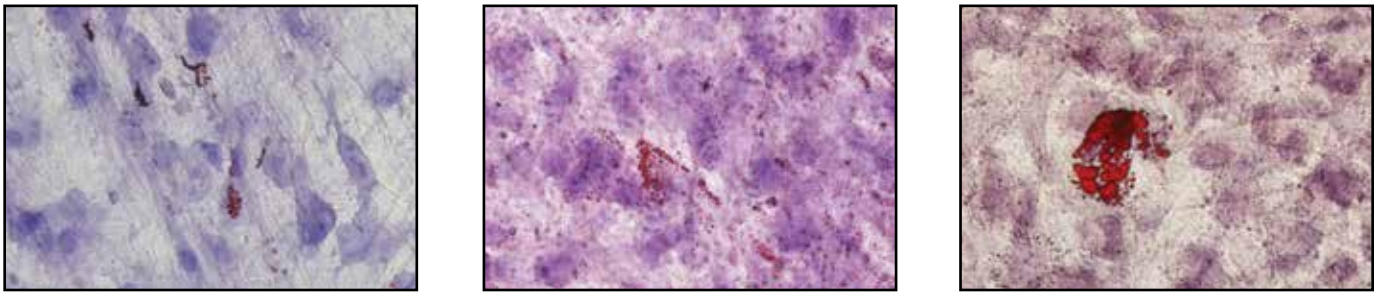
2 weeks ***4 weeks*** ***8 weeks***

Figure 3: Positive Alizarin Red S (red stain) and von Kossa staining (black deposits) on explanted cells from Trinity Evolution tissue at 2, 4 and 8 weeks in culture.

ADIPOGENESIS

Explanted cells from Trinity Evolution tissue were also evaluated for their ability to undergo adipogenic differentiation. Confluent explanted cells from passage 1 expansion were trypsin-treated and re-seeded into 24-well tissue culture plates. The cells were induced with adipogenic stimulatory medium[4,7] for up to 12 weeks with media exchange every third or fourth day. Confluent monolayers were stained with Oil Red O followed by a Hematoxylin & Eosin (H&E) counter-stain at 4, 6 and 12 weeks. Positive red staining of the Oil Red O dye indicated lipid vacuole formation of adipogenically differentiated cells, while the cell nuclei were distinguished by the H&E counter-staining. *Figure 4* shows the positive adipogenic staining, which increased over time in culture (images were taken at 40x magnification). These observations highlight the ability of the explanted cells to differentiate into the adipogenic lineage.

Oil Red O Staining



2 weeks ***4 weeks*** ***8 weeks***

Figure 4: Positive Oil Red O (red staining) on explanted cells from Trinity Evolution tissue over 4, 6 and 12 weeks.

DISCUSSION

The data generated in this study demonstrates that the cells derived from explants of Trinity Evolution tissue have the ability to migrate out of the tissue, proliferate, and exhibit typical characteristics of MSCs. The explanted cells were also shown to have the functional ability to successfully undergo osteogenic and adipogenic differentiation over time. In particular, significant mineralization was observed in the osteogenic cultures by 8 weeks. Collectively, these *in vitro* findings suggest that the cells within Trinity Evolution tissue have the potential to grow out of the tissue, multiply, and form bone following implantation.

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