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Purmorphamine Promotes Matrix Mineralization and Cytoskeletal Changes in Human Umbilical Cord Mesenchymal Stem Cells

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Abstract: Human Umbilical Cord Mesenchymal Stem Cells (hUCMSCs) were subjected to *in vitro* osteogenic differentiation using a novel combination of signaling molecules including BMP-2 and purmorphamine. Differentiation outcomes were assessed by calcein staining and by microscopic examination of the cytoskeleton. Calcein staining showed appreciable degree of calcium mineralization in cell culture, and changes in the morphological attributes of differentiating cells were observed vis-a-vis the actin cytoskeleton. Finally, positive calcein staining, altered cytoskeletal profile, and stress fiber formation in treated cells demonstrated, for the first time, a potentially synergistic interplay between BMP-2 and the hedgehog agonist, purmorphamine.
This study lends support to the notion of combining small doses of potent molecules that can act as safe, less toxic inducers of osteogenic differentiation of human umbilical cord mesenchymal stem cells with respect to bone regeneration.

Keywords: purmorphamine, BMP-2, lithium chloride, hUMSCs (human umbilical cord mesenchymal stem cells, fibroblast, osteogenic differentiation, cytoskeletons, actin stress fibers.

1 Introduction

Chemical induction of osteogenic differentiation has been shown to take place via modulations of signaling pathways that trigger genes on and set into motion a cascade of protein expression changes that promote matrix mineralization (1-2,8). Previous experiments have confirmed changes in the actin cytoskeleton prior to osteogenic differentiation in various cell lines. Interactions between actin and myosin II, myosin II in turn being regulated by the Rho A /ROCK pathway, have been

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implicated as the pivotal events in driving stress fiber formation and isometric tension generation within the cell (1,5, 8). In this study, we examined the induction of ostegeonesis in human umbilical cord mesenchymal stem cells using purmorphamine, a tri-substituted purine analog, and BMP-2 (bone morphogenic protein) by (a) measuring matrix mineralization using calcein staining and (b) by visualizing the changes in actin cytoskeleton using microscopy (3-4, 6-8).

2 Materials and Methods

Ethical Approval: These experiments were approved by and carried out according to the regulations outlined by the IRB at the University of Kansas.

Calcein affording yellow-orange fluorescence (C0875) was obtained from Sigma and it had wavelengths of excitation and emission at 470 and 509 nm respectively. Green fluorescenct Alexa Flour 488 phalloidin probe (Ex_{495}/Em_{518}) was purchased from Molecular Probes (Catalog number 12379).

HUCMSCs (human umbilical cord mesenchymal stem cells were harvested from placenta obtained via informed consent as per the protocols approved by the Committee on Ethics(IRRB) at the University of Kansas. Cells were grown in low glucose Dulbecco's Modified Eagle's Medium, 10 % FBS, and penicillin/streptomycin (PS). Then, hUCMSCs were seeded at a density of $1 * 10^4$cells per cm^2. Cells were then grown to confluence in a 12-well tissue culture-treated plate. Next, mesenchymal stem cells were treated with purmorphamine or vehicle (dimethly sulfoxide) in the presence of standard osteogenic media for 2-6 weeks. Cells were incubated at 37 degrees under humid conditions with 5% CO2. The effects of purmorphamine on cell viability and cell growth (IC-50 determination) were characterized, cell nuclei and the cytoskeleton were examined, and the formation of extracellular calcium measured qualitatively by calcein staining.

Calcein staining:

Using our protocol for osteogenic differentiation, hUCMScs were incubated with purmorphamine for

2 and 4 weeks. After removing the media, the cells were washed with PBS twice. Next, 0.0004 g Calcein was dissolved in 50 ml of cell culture media. The cells were incubated overnight in 1ml of this Calcein red solution overnight according to the protocol of Hale et al. (12). The stained cells were washed with distilled water to flush out excess dye, fresh media was added, and the cells were examined under a fluorescence microscope with appropriate filter sets (NikonTS 100).

Actin visualization:

Drug –treated hUCMScs were washed twice with PBS, fixed with 4% paraformalde-

hyde for 10 minutes, and permeabilized with 0.1 % Triton X. Nonspecific binding was blocked with BSA incubation. The fixed cells were then incubated in fluorescently- conjugated phallodin antibody(Green Fluorescent Alexa Flour 488 probe, Molecular Probes)overnight at 37 degrees C for 30 minutes. To prevent quenching, cells were kept in the dark. Next morning, the cells were examined under a fluorescence microscope with appropriate filter sets (Nikon TS 100).

3 Results & Discussions:

The effects of purmorphamine on cell viability and cell growth (IC-50 determination) were characterized: cell growth was reduced with 2.5 uM purmorphamine and the cell nuclei appeared healthy (unpublished data). Compared to controls, calcium levels did increase in cells treated with purmorphamine as shown by calcein staining at 4 weeks; cells are extending lamellopodia (double-headed arrow), and calcium deposits can be seen around the cells, as shown by the arrows in Fig 2 B. (Fig. 2Aand B). Examination of the cytoskeleton in human umbilical cord mesenchymal stem cells revealed healthy actin fibers (Fig. 1A).

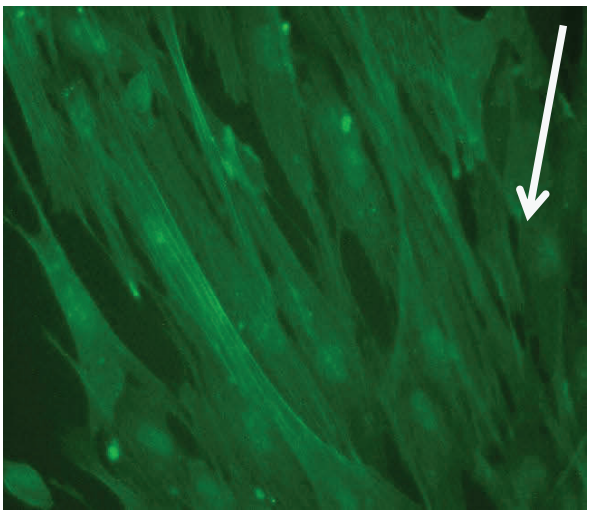

Figure 1A: Control human umbilical cord mesenchymal stem cells stained for actin at 2 weeks. There are more fibers toward the periphery of the cells(arrowhead) as compared to the treated cells in Fig 1B. Control cells have elongated, fibroblast-like appearance as compared to the cuboid-like shape in the treated cells.

We also observed the formation of actin-rich membrane ruffles and noticed that

the ends of the actin filaments were undergoing rapid growth, in our treated cells (Fig.1B). Additionally, the treated cells assumed a more cuboidal or spherical shape(line in Fig 1B) as compared to the fibroblast-like, elongated appearance that control hUMSCs exhibited in Fig. 1A(9). Moreover, purmorphamine treatment appeared to have reduced the number of long actin fibers (Fig1B) and these cells had less fibers towards the periphery of the cells(Fig. 1B). Furthermore, the distribution of actin fibers in the hUCMSCs that were undergoing osteogenic differentiation was different from hUCMSCs grown in growth media; upon close examination of the actin fibers, it can be seen that the actin fibers in the control cells were oriented parallel to the longitudinal axis whereas the actin fibers in treated cells, that were undergoing osteogenesis, were displaying a random, non-parallel alignment as seen in Fig 1A and 1B. In addition, cells undergoing osteogenesis showed greater extensions of focal adhesions (Fig 1B). Our observations, with regards to the orientation of actin fibers as well as the formation of focal adhesions, are in accord with those of Born et al (9-11).

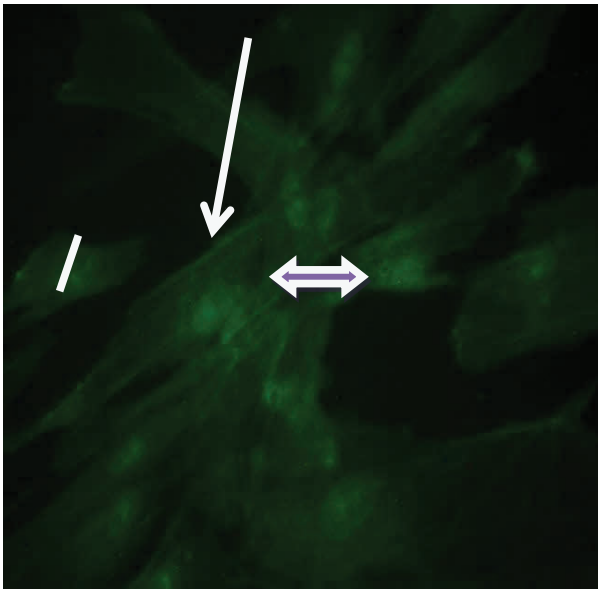

Figure 1B: human umbilical cord mesenchymal stem cells treated with 2.5 uM Purpmorphamine and stained for actin at 2 weeks . Actin fibers are shorter in length compared to control, have actin-rich membrane ruffles (arrowhead), and the ends of actin fibers are growing(double-headed arrow).

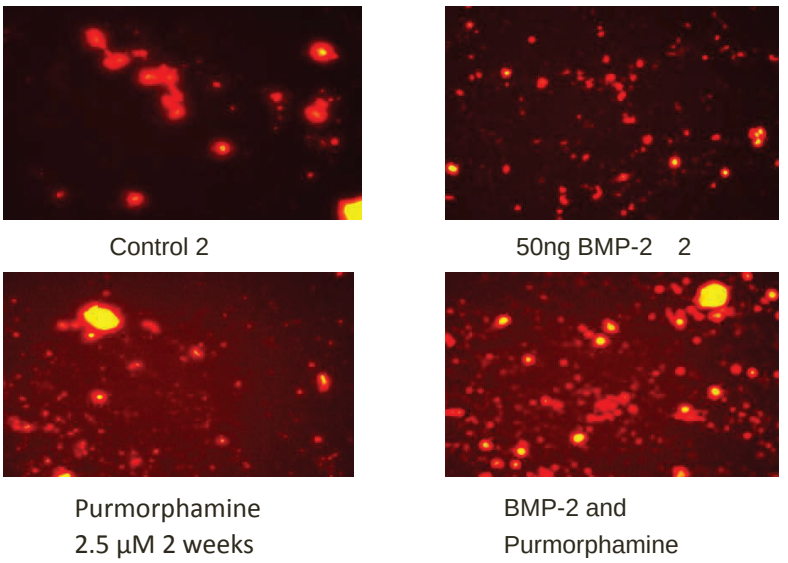

Figure 2A: Calcein detection of mineralization at 2 weeks.

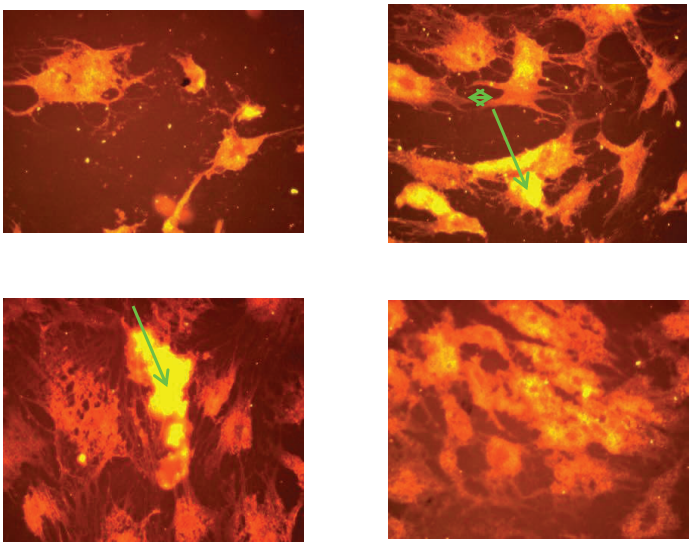

Figure 2B: Calcein detection of mineralization at 4 weeks. Cells are extending lamellopodia(double-headed arrow), a network of collagen fibrils is see undergirding the cells, and calcium deposits can be seen around the cells(arrows).

4 Conclusion

Our study shows for the first time that a synergistic combination of purmorphamine and BMP-2 induces osteogenesis related processes such as actin stress fiber reorganization, bone mineralization, and collagen fiber formation in human umbilical cord mesenchymal stromal cells (hUCMSCs). Mai et al have found similar results in a murine cell line(11).

Our observations also highlight a greater need for the examination of the effects of other small molecules, such as lithium, on bone formation, since lithium has been used as an antipsychotic drug in many patients, who will live for many years to come.

As a whole, our results indicate that purmorphamine could be used as an inducer of osteogenic differentiation in human mesenchymal stem cells. Since human umbilical cord mesenchymal stromal cells happen to be less tumorogenic and tend to escape alloantigenic recognition, future studies including phenotypic analysis of cells are warranted to further validate our findings.

Our results also validate, albeit to a small degree, the concept of using synergistic combination of potent molecules in differentiation experiments aimed at elucidating cellular biomechanics.

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