

Cell communication and tissue engineering

Ricardo A. Rossello and David H. Kohn*

Departments of Biologic and Materials Sciences, and Biomedical Engineering; University of Michigan; Ann Arbor, MI USA

Gap junction intercellular communication (GJIC) is ubiquitous in the majority of cells and is indispensable for proper development and function of most tissues. The loss of gap junction mediated cell to cell communication leads to compromised development in many tissues and organs, and also facilitates tumorigenesis and autonomous cell behavior in cancerous cells. Because cells embedded in an extracellular matrix constantly interact through gap junctions to coordinate normal tissue functions and homeostasis, our group hypothesized that increasing cell to cell communication, via genetically engineering cells to overexpress gap junction proteins, could improve cell signaling and increase differentiation in interior regions of engineered tissue equivalents. In a recent paper,¹ we presented a platform to regenerate full 3D equivalents of engineered tissue, providing a strategy to overcome a barrier in regenerative medicine. These findings suggest that both targeted delivery and cell-based strategies can be used as treatments to enhance communication in 3D living tissue.² In this addendum, we address the effects of extracellular calcium (Ca^{2+}_e) on intracellular calcium (Ca^{2+}_i), GJIC and osteogenic differentiation under conditions in which bone marrow stromal cells (BMSCs) also exhibit higher cell-to-cell communication. As a key secondary messenger in many biological processes, the levels of Ca^{2+}_e and Ca^{2+}_i play a role in cell differentiation and may be a tunable signal in tissue regeneration. Higher cell-to-cell communication was achieved by both genetically engineering cells to overexpress connexin 43 (Cx43) and by a high density cell seeding technique, denoted micromass

seeding (MM). The results presented in this addendum show that the intensity and duration of a second messenger, like calcium, can be augmented in a platform that enables higher cell-to-cell communication. The ability to modulate calcium signaling, combined with our previous approaches to modulate GJIC, may have an impact on tissue regeneration and therapies for communication incompetent cells, such as those associated with heart disease and certain types of cancer.

Control of Cell-Cell Communication: A New Platform for 3D Tissue Regeneration

Direct cell to cell communication through connexin 43 (Cx43) generated gap junctions allows for the rapid propagation of ions, secondary messengers and metabolites in the majority of cells. Because of the ubiquitous nature of Cx43 throughout most vertebrate cell types, this protein provides a signaling platform that enables cells to communicate.³ Several Cx43 knockout models exhibit developmental abnormalities and compromised function of a number of tissues, including heart and bone, suggesting that cell to cell communication through gap junctions is essential for proper development.^{4,5} Furthermore, the cell autonomy that characterizes cancer cells may be due to decreased expression of Cx43.⁶ In many cell-based tissue engineering strategies, the ability to regenerate tissue in 3 dimensions is limited by compromised transport and differentiation of cells in the interior of cell-seeded extracellular matrix analogues (scaffolds). Based on the importance of cell to cell communication and the functions of

Key words: connexin 43 (Cx43), gap junction intercellular communication (GJIC), calcium, micromass cultures, bone

Submitted: 08/17/09

Accepted: 08/18/09

Previously published online:

www.landesbioscience.com/journals/cib/article/9863

*Correspondence to:

David H. Kohn; Email: dhkohn@umich.edu

Addendum to: Rossello RA, Wang Z, Kizana E, Krebsbach PH, Kohn DH. Connexin 43 as a signaling platform for increasing the volume and spatial distribution of regenerated tissue. *Proceedings of the National Academy of Sciences* 2009; 106:13219–24; PMID: 19628695; DOI: 10.1073/pnas.0902622106.

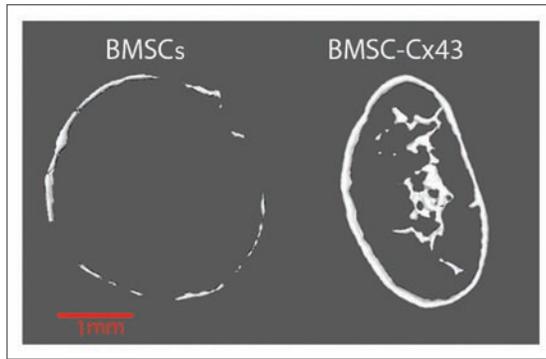


Figure 1. Comparing bone regenerated by transplanted bone marrow stromal cells (BMSCs) and BMSCs overexpressing Cx43. This image shows micro-computed tomography renderings of bone regenerated subcutaneously two months after cell transplantation. In the bone regenerated from BMSCs there is peripheral bone formation, but relatively low amounts of core tissue formation. Cells overexpressing Cx43 regenerated more bone and bone penetrated farther into the core area, suggesting a platform for tissue regeneration.

Cx43 in normal and pathological development, we hypothesized that enhancing gap junction intercellular communication (GJIC) would enable cells seeded in a 3D scaffold to be more highly networked, allowing more efficient transmission of differentiation cues to cells throughout the complex 3D environment.

To test this hypothesis, we used bone as a model tissue,¹ because of the inherent limitation in achieving spatially uniform bone regeneration of sufficient thickness to be clinically meaningful.⁷ Our results¹ showed that bone marrow stromal cells (BMSCs) transduced with a Cx43 lentivirus exhibited increased

GJIC, increased magnitude and spatial distribution of osteogenic differentiation markers and increased volume fraction of bone formed in-vivo relative to non-transduced BMSCs (Fig. 1). Importantly, differentiation in-vitro and tissue regeneration in-vivo were significantly greater in the core regions of the scaffolds (0.5–3 mm away from nutrients) containing cells that were engineered to overexpress Cx43, relative to core regions of scaffolds containing non-transduced BMSCs.¹ Furthermore, there was no difference in GJIC and osteocalcin mRNA expression between peripheral regions (0.5 mm away from nutrients) and core regions in constructs containing cells overexpressing Cx43, compared to significant decreases in these markers in the core of constructs containing non-transduced BMSCs.¹ Taken together, these results indicate that spatial gradients in signals that inhibit regeneration of thick tissue equivalents can potentially be overcome through control of gap junctions.

Effect of Increased GJIC on Secondary Signals

GJIC also enables the distribution of signals triggered by a secondary stimulus, either electrical, mechanical or biological in nature, between neighboring cells.² To test the effect of higher GJIC in tandem with a secondary stimulus, cells were induced to overexpress bone morphogenetic protein 7 (BMP7).⁸ This growth factor was chosen because it has potent osteoinductive effects, but does not modulate the expression of Cx43.⁹ Enhanced GJIC coupled with enhanced expression of BMP7 increased the osteogenic effect relative to BMP7 alone,¹ suggesting that the overexpression of Cx43 synergistically enhances the effects of a secondary stimulus, possibly by increasing its spatially distributed differentiation potential. Furthermore, the overexpression of Cx43 increased the expression of other soluble factors, suggesting that the enhanced differentiation can occur by means of direct (formation of more channels) and indirect (secretion of higher levels of soluble factors that induce differentiation) effects of Cx43.¹

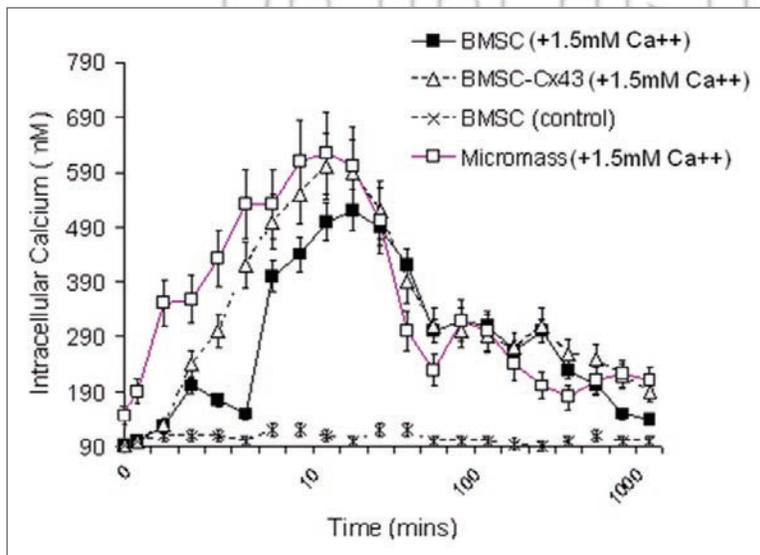


Figure 2. Intracellular calcium profiles. Intracellular calcium was tracked over time to determine the response to a higher extracellular calcium environment. BMSCs, BMSCs overexpressing Cx43 and BMSCs seeded in micromasses were exposed to a 1.5mM increase in extracellular calcium concentration at $t = 1$ min. The intracellular level of calcium in micromass seeded cells was higher than all other groups at $t = 0$, prior to the increase in extracellular calcium ($p < 0.001$). Peak levels of intracellular calcium (10–50 min after exposure to the increase in extracellular calcium) in micromass and Cx43 transduced cells were higher than in BMSCs. After 1000 min, the intracellular calcium levels in BMSCs, BMSCs exposed to elevated calcium and BMSCs seeded in micromass reverted back to their baseline levels, whereas the Cx43 transduced cells had a significantly higher level of intracellular calcium compared to its initial level ($p < 0.001$).

Effects of Increasing Cell-Cell Communication and Extracellular Calcium

In addition to investigating the effects of a soluble growth factor coupled with a higher cell-to-cell communication platform, we investigated the effects of increasing the level of soluble extracellular calcium (Ca^{2+}) coupled with increased cell-cell communication in BMSCs. In the experiments described in this addendum, we investigated the effect of soluble calcium on intracellular Ca (Ca^{2+}), GJIC and osteogenic differentiation in BMSCs, BMSCs transduced to overexpress Cx43, and BMSCs cultured in micromasses (MM). Calcium-phosphate (Ca/P) templates may be physiologically favorable for cell transplantation, especially in mineralized tissue engineering applications, because they mimic the mineralized extracellular environment and can release ions that aid in the regeneration of bone.¹⁰ Therefore, in addition to surface-mediated signals, soluble calcium ions may also regulate cell differentiation. The capacity in which cells communicate with one another may facilitate ion transfer and may play a role in regulating the concentration of intracellular calcium. A high density seeding technique, denoted micromass cell seeding (MM), also increases cell seeding capacity, nutrient transport and cell-cell communication.¹⁰ Therefore, in addition to increasing cell to cell communication via endogenously controlling Cx43 expression, an exogenous strategy of increasing cell to cell communication was also used.

Cells were cultured in 12-well plates. Normal media (α -MEM, 10% FBS, 100 $\mu\text{g}/\text{ml}$ penicillin G, 100 IU/ml streptomycin) was substituted with one containing an additional 1.5 mM Ca via the addition of CaCl_2 . Ca^{2+} levels were quantified by imaging the calcium levels with the Ca^{2+} sensitive dye Fura-2. Prior to increasing Ca^{2+} , cells were incubated with Fura-2AM in media and washed with PBS. Ca^{2+} was measured in BMSCs, MM seeded BMSCs and BMSCs transfected with Cx43 in a control environment (1.5 mM baseline concentration at time zero) and after increasing the extracellular calcium concentration by 1.5 mM (total 3 mM).

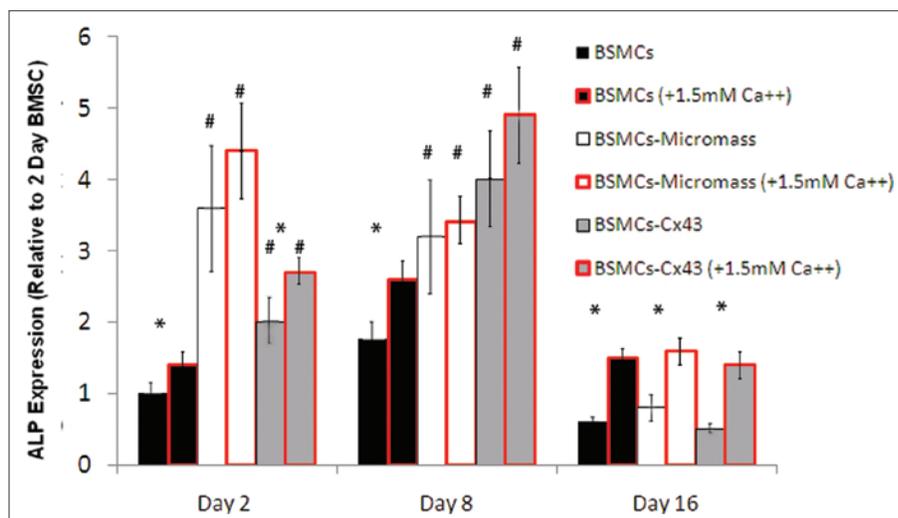


Figure 3. A higher calcium environment leads to significantly higher expression of ALP in BMSCs at all times. Higher cell-to-cell communication (via both Cx43 overexpression and micromass seeding) leads to significantly higher levels ALP expression at early time points (2 and 8 days). Bars that are bordered by a red line indicate a higher calcium environment; * denotes group pairs where a higher calcium environment produces significantly higher levels of ALP expression; # denotes significantly higher expression over analogous BMSC-only environment (normal or high calcium).

The Ca^{2+} was monitored for 16 hrs, GJIC was assessed for all groups by lucifer yellow transfer to adjacent cells after 10 minutes. Real-time PCR was used to detect the effects of increased extracellular calcium with Cx43 overexpression and MM culturing on ALP expression (at 2, 8 and 16 days after induction of differentiation; normalized to 18 s expression).

For control BMSCs, as well as BMSCs manipulated endogenously and exogenously to enhance cell to cell communication, the Ca^{2+} level increased 4 to 6-fold after exposure to higher calcium in the media (Fig. 2). Micromass cultured cells had a significantly higher initial Ca^{2+} level (140 ± 5.5 nM, $p < 0.001$) than all other groups (BMSCs: 89.1 ± 3.2 nM; BMSC-Cx43: 91.4 ± 3.1 nM) at time zero, before being exposed to the 1.5 mM increase in Ca^{2+} . The Ca^{2+} for all cells exposed to the increase in extracellular calcium peaked at 10–50 minutes, then decreased with time. All cells returned to their basal level of Ca^{2+} at 16 hrs, except the Cx43-cells. Cells overexpressing Cx43 exhibited a significantly higher Ca^{2+} level at 16 hrs (193 ± 18.3 nM) relative to time zero ($p < 0.001$), suggesting more Ca^{2+} retention with this mode of increasing cell to cell communication. Increasing Ca^{2+} did not affect GJIC (control BMSCs

= $26.1 \pm 3.9\%$ of cells vs. BMSCs with 1.5 mM Ca = $29.9 \pm 4.1\%$; $p > 0.05$). However, Cx43 transfected cells had significantly higher GJIC ($43.2 \pm 5.1\%$) than MM ($33.1 \pm 7.3\%$, $p = 0.031$), control BMSCs ($p < 0.001$) and BMSCs with 1.5 mM Ca ($p < 0.001$).

Significantly more ALP activity occurred in BMSCs exposed to an elevated calcium environment ($p < 0.001$ at all times) than in BMSCs cultured in the control environment (Fig. 3). ALP expression was also enhanced by MM seeding and Cx43 overexpression at 2 and 8 days, and this effect was significant in both normal and elevated calcium environments. Synergistic effects between elevated cell-cell communication and elevated calcium were also observed, similar to the synergistic effects observed between GJIC and expression of a secondary growth factor.¹ At 16 days, when ALP expression declined from its peak, expression was still relatively greater in the presence of elevated calcium.

Conclusions and Future Directions

Taken together, these results show that higher Ca^{2+} , such as in the environment created during dissolution of Ca/P

biomaterials, increases Ca^{2+}_i in cells, and enhances differentiation. Furthermore, the concentration of soluble intracellular calcium can be augmented by enhancing GJIC. Increasing cell-cell communication enhances the intensity of the calcium burst and transiently enhances baseline intracellular calcium levels. Controlling the magnitude and duration of intracellular calcium demonstrates that GJIC can be used to modify the magnitude and time course of another secondary messenger.

These calcium studies, along with our previous data,¹ suggest that increasing gap junction intercellular communication via overexpression of Cx43 can be, on its own, a tool to overcome limitations inherent in cell to cell communication and regeneration of tissue in 3 dimensions. These studies also showed that cell to cell communication can be a mechanism to

augment other secondary messengers and signals that can lead to the regeneration of larger and more uniform volumes of tissue. Enhancing cell-to-cell communication may also impact therapies based on calcium signaling.

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