Co-culture of cardiac fibroblasts and myocytes enhances functional assembly of engineered myocardium

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Introduction: Native myocardium consists of several cell types of which 1/3 are myocytes and most of the non-myocytes are fibroblasts [1]. Cardiac myocytes are the largest cells in the myocardium, they occupy ~90% of the volume and are responsible for synchronous contractions of the ventricles. The main roles of cardiac fibroblasts are to secrete the components of the extra-cellular matrix (ECM) mainly collagen (Burlew 2002) and to transmit mechanical force by the receptor mediated connections to the ECM [2]. The exact composition of the ECM is regulated by a cross-talk between myocytes and fibroblasts [2]. Recent studies demonstrated that cardiac fibroblasts can propagate electrical stimuli over the distances on the order of 100μm via gap junctional communications [3].

By analogy with monolayer culture in which fibroblasts are removed to prevent overgrowth, early attempts to engineer myocardium utilized cell suspensions enriched for cardiac myocytes (~80-90% cardiac myocytes) by preplating [4, 5]. The engineered cardiac constructs exhibited markers of cardiac differentiation and were able to propagate electrical signals over several millimeters. However, cells in these constructs did not align in parallel as in the native myocardium and neither the force of contraction nor the macroscopic contractile response were investigated.

We hypothesized that the co-culture of cardiac fibroblasts and cardiac myocytes will enhance functional assembly of the engineered cardiac constructs by enabling scaffold remodeling and active cross-talk between cells.

Methods: Cells from the neonatal rat ventricles were divided into three groups: unseparated cells (US), cardiac fibroblasts (CF) (obtained by preplating and expansion in T75 flasks) and enriched cardiomyocyte group (CM) (consisting of cells that remained unattached after 75 min preplating). The cell fractions were applied to the poly(glycerol sebacate) scaffolds (5mm diameter 2mm thickness) using Matrigel^{TM} separately (CM only), at the same time (US only) or serially (CF pretreatment for 5 days followed by addition of CM-pretreated group). Total cell number added was $3 \cdot 10^6$ in all groups. After 7 days of cultivation in spinner flasks the constructs were harvested and tested for ability to contract synchronously when placed between two parallel electrodes and stimulated with a cardiac stimulator. Amplitude of contraction, excitation threshold (minimum voltage necessary to induce contractions) and maximum capture rate (maximum beating frequency) were determined. Protein, DNA content and glucose metabolism were measured. Expression of cardiac (troponin-I, α-actin, connexin-43) and fibroblast markers (vimentin, prolyl-4-hydroxylase) was assessed by immunohistochemistry and Western blots. Distribution of myocytes, non-myocytes and fibrillar collagen was assessed my Mason's Trichrome staining. The ratio of fibroblasts to myocytes in the original cell suspensions was quantified by FACS and in the constructs it was determined by morphomety from double stained sections.

Results: The presence of fibroblasts improved amplitude of contraction in unseparated and pretreated groups as expected with the amplitude of contraction (AC) 5 times higher in the pretreated compared to the cardiomyocyte only group (Table 1). The pretreated group also had the lowest excitation threshold (Table 1). The superior functional properties of the

pretreated group can be explained by construct composition and morphology. The pretreated group significantly higher DNA content than the US and CM groups and comparable to the adult heart resulting in a low excitation threshold (Table 1). Protein content and the glucose consumption rate of the pretreated group were ~2 times higher compared to the other groups, enabling high amplitude of contraction (Table 1).

Histology revealed the presence of cells in the 100-200μm thick surface layer of engineered tissue (Figure 1). In the unseparated group, myocytes were aligned in parallel in short domains separated by large cell-free or fibroblasts only domains. In the cardiomyocyte only group, relative ratio of fibroblasts/myocytes was similar as in the pretreated group but the myocytes failed to elongate and organize in parallel. The pretreated group exhibited compact layers of elongated myocytes aligned in parallel, with fibroblasts located preferentially in the surface layer. Staining for prolyl-4-hydroxylase, revealed the presence of collagen depositing fibroblasts in the pretreated group. Collagen depositing fibroblasts were absent in the cardiomyocyte group and very rare in the unseparated group. Mason's trichrome staining revealed compact regions of myocytes in surrounded by collagen domains in pre-treated group only.

Conclusion: Taken together, the data indicate that the presence of actively secreting fibroblasts is essential for improved functional assembly of engineered myocardium. Improved tissue properties in the pretreated group can be explained by scaffold remodeling and deposition of extracellular matrix and soluble factors by fibroblasts during pretreatment. This created a cardiac-like environment supportive of tissue assembly upon addition of myocytes. Although, fibroblasts were present in the unseparated and cardiomyocyte group, their secretory ability was low and insufficient for scaffold remodeling. Areas of future work involve determination of molecular mechanisms involved in the myocyte-fibroblasts cross-talk during tissue assembly in 3D.

Table 1. Contractile and biochemical properties of engineered cardiac tissue. Data expressed as ave±se (n=3-10).*Significantly different than pretreated group by Tukey's test in conjunction with one way ANOVA.

Property	CM	US	Pretreated
ET [V]	2.99±0.11	3.20±0.15*	2.73±0.09
MCR [bpm]	188±13	194±19	207±10
AC [% area change]	0.17±0.03*	0.57±0.22	0.67±0.13
DNA/ww [μg/mg]	0.30±0.33*	0.33±0.07*	0.66±0.06
Prot/ww [μg/mg]	20.3±1.2*	19.7±2.5*	39.4±4.2
Glucose consumption Rate [μmol/cons/hr]	0.08±0.02	0.11±0.03	0.18±0.06

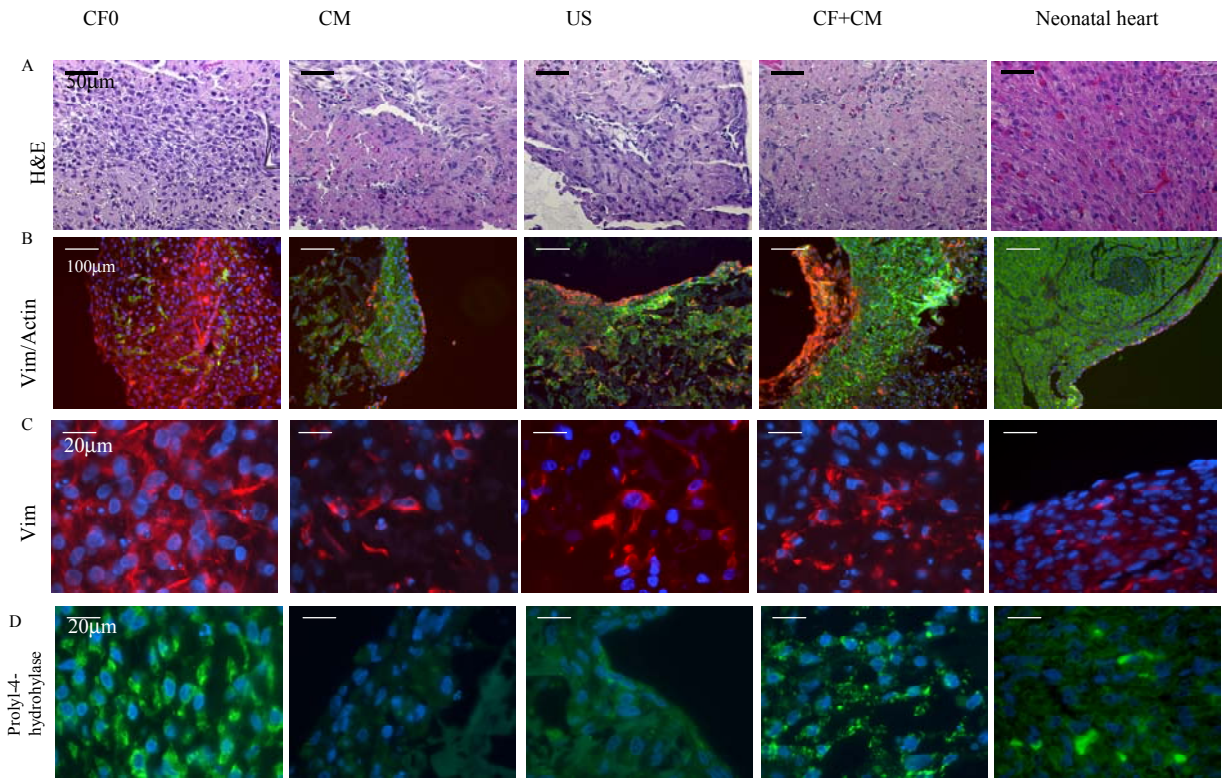

Figure 1 Tissue morphology and expression of cardiac and fibroblast markers. Constructs were paraffin embedded and face sectioned within first 100µm. A) Hematoxylin and Eosin staining B) Vimentin and actin double staining to identify fibroblasts and myocytes respectively C) Vimentin staining at higher magnification to identify fibroblast morphology D)Prolyl-4-hydroxylase to identify collagen secreting fibroblasts

1. Nag, A.C., Study of non-muscle cells of the adult mammalian heart: a fine structural analysis and distribution. Cytobios, 1980. 28(109): p. 41-61.
2. Sussman, M.A., A. McCulloch, and T.K. Borg, Dance band on the Titanic: biomechanical signaling in cardiac hypertrophy. Circulation Research, 2002. 91(10): p. 888-898.
3. Gaudesius, G., et al., Coupling of cardiac electrical activity over extended distances by fibroblasts of cardiac origin. Circulation Research, 2003. 93(5): p. 421-428.
4. Papadaki, M., et al., Tissue engineering of functional cardiac muscle: molecular, structural and electrophysiological studies. American Journal of Physiology: Heart and Circulatory Physiology, 2001. 280(Heart Circ. Physiol. 44): p. H168-H178.
5. Bursac, N., et al., Cardiac muscle tissue engineering: toward an in vitro model for electrophysiological studies. American Journal of Physiology: Heart and Circulatory Physiology, 1999. 277(46): p. H433-H444.