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Research Article

Zebrafish keratocyte explant cultures as a wound healing model system: Differential gene expression & morphological changes support epithelial–mesenchymal transition

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ARTICLE INFORMATION

Article Chronology:
Received 16 January 2013
Received in revised form
19 March 2013
Accepted 20 March 2013

Keywords:
Collective cell migration
Epithelial wound healing
Primary cultures
EMT
Transcription networks

ABSTRACT

The control of collective cell migration of zebrafish keratocyte sheets in explant culture is of interest for cell migration and epithelial wound healing and depends on the gene expression profile. In a zebrafish genome array, ~17.5% of the probe sets were differentially expressed greater than two-fold ($p \leq 0.003$) between 1 and 7 days of explant culture. Among the differentially expressed genes were a variety of wound healing-related genes and many of the biomarkers for epithelial–mesenchymal transition (EMT), including a switch from keratin and E-cadherin to vimentin and N-cadherin expression and several EMT-related transcription factors were found to be differentially expressed. Supporting evidence for EMT is seen in both morphological change and rearrangement of the actin cytoskeleton and in expression of cadherins during explant culture with a visible disassembly of the cell sheet. TGFβ1 and TNFα expression were analyzed by qPCR at various time points and peak differential expression of both cytokines occurred at 3 days, indicating that the EMT process is ongoing under conditions routinely used in the study of fish keratocyte motility. These data establish that an EMT process is occurring during zebrafish keratocyte explant culture and support the use of this system as a wound healing model.

Introduction

Because of its rapid, smooth gliding motion, while maintaining a uniform shape, speed, and direction, the fish keratocyte has been a powerful model system to study the mechanisms of cell motility at the single-cell and mathematical modeling level [1–4]. In these studies, keratocytes are cultured by placing a scale plucked from an anesthetized fish in medium and the resulting cell sheets are then dissociated and replated for the study of single-cell motility. However these cells only retain their unique morphology and motile characteristics for 1–3 days [5] so that new cultures must be regularly established. To date, these changes in motility and morphology over time in explant culture remain largely uncharacterized. Instead of focusing on individual keratocytes, our laboratory focuses on the mechanisms that direct the collective cell migration of zebrafish keratocyte cell sheets during explant

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http://dx.doi.org/10.1016/j.yexcr.2013.03.036

culture. When the explant culture is viewed as an epithelial wound healing model, in which the cells migrating away from the explant as they would across the provisional matrix of a wound bed to reestablish an intact epithelium, the changes in keratocyte motility and morphology over time in explant culture may be part of the normal processes of epithelial wound healing seen in other systems.

As the primary epithelial cell of their respective species, the fish keratocyte is the functional equivalent of the human keratinocyte. However, while studies on fish keratocytes use 1–3 day primary cultures, most studies on human epithelial wound healing are performed with either transformed cell lines or primary cultures which have been cultured long-term, a process which leads to distinct differences from freshly isolated cells [6–10]. Yet the functional relationship between human keratinocytes and fish keratocytes is suggestive of a possible molecular relationship governing the behavior of these two cell types. Specifically, can the migration of keratocyte cell sheets during explant culture be seen as a wound healing model system?

Epithelial to mesenchymal transition (EMT) is a well-defined, integral part of many wound healing processes. EMT encompasses the loss of epithelial cell polarity and adhesive junctions with the increased migration and concurrent remodeling of ECM characteristic of mesenchymal cells. Broadly defined, EMT describes, at the cellular level, many processes involved in the several overlapping phases of wound healing. In vivo, wound healing is typically divided into sequential but overlapping phases including hemostasis, inflammation, proliferation, and remodeling. The inflammatory phase is partially recapitulated in the keratocyte system although fish do not bleed when scales are harvested (so that hemostasis is not immediately a consideration). In addition, the proliferative and remodeling phases of epithelial wound healing include many of the cellular hallmarks of EMT: collagen deposition, migration of cells in reepithelization, and tissue remodeling. Thus, the EMT refers to changes at the cellular level, within the context of a small number of neighboring cells, and is therefore more relevant to our in vitro model than in vivo systems. Recent data have documented increased expression of TGFβ1 and its receptor during keratocyte explant culture, defining a role for this cytokine in increasing keratocyte motility [11]. This supports the supposition that EMT is occurring in the keratocyte explant system as TGFβ1 is closely associated with triggering EMT [12–15].

As recent reviews have collated changes in gene expression involved in EMT [15–18], we have looked for evidence of differential expression of these EMT-related genes in the keratocyte explant culture system. Initial microarray data were analyzed to identify which zebrafish homologs of EMT associated genes that were differentially expressed between 1 and 7 day explant cultures. These data substantiate a loss of epithelial and a resulting gain of mesenchymal markers, a finding that is substantiated by the morphological change which occurs during this time. qPCR experiments on differential expression of TNFα and TGFβ1 indicate that peak expression of these two cytokines occurs between 1 and 7 days, a finding that may explain the absence of differential expression for some wound healing-associated gene products. Taken together, the data presented support a role for EMT in keratocyte explant culture and support the proposition that this system is an epithelial wound healing model.

Materials & methods

Zebrafish keratocyte primary explant culture

Keratocytes from anesthetized AB wild type zebrafish (Zebrafish International Resource Center, Eugene OR, USA) were cultured using standard methods in RPMI 1640 medium with Hepes pH 7.4, 10% fetal bovine serum (FBS), 100 μg/ml kanamycin, and 50 μg/ml gentamicin (VWR, Radnor PA, USA) and grown at 28 °C in 5% CO_2.

Microarray analysis

RNA was isolated from keratocytes in explant culture using RNAPure (GenHunter, Nashville TN, USA) following manufacturer's instructions. Affymetrix zebrafish genome array (Santa Clara CA, USA) analysis was completed by Expression Analysis (Durham NC, USA) and included amplification, labeling, hybridization, and analysis of the output by two-group comparisons with permutation analysis for differential expression (PADE). Tables were generated from array results using NetAffx annotation files (release 32) from Affymetrix. In the event of multiple probe sets for one reference protein, the probe set with no annotations referring to cross hybridizing matching probes as well as the most significant p-value was selected. In the event of equal p-values (e.g. both 1.000 or 0.000), the probe set with the most conservative fold change in expression is reported. Probe sets with *p*-values between 0.0030 and 0.9975 were deemed unchanged. Signal levels, log ratio changes, and PADE generated *p*-values for all probe sets discussed in the manuscript are included in supplemental Table S1. The microarray data was validated by performing qPCR on 8 genes, selected from the most induced, most decreased, and least changed genes in the microarray results, with primers used and results shown in Supplementary Table S2.

qPCR of TGFβ1 and TNFα mRNA

RNA was isolated as above followed by cDNA synthesis with Superscript Vilo (Invitrogen, Carlsbad CA, USA) and qPCR performed with SYBR Green (Express qPCR with Premixed ROX, Invitrogen CA, USA) and custom primers (IDT DNA, Coralville IA, USA) using an Applied Biosystems Step One Plus thermocycler. The primers used were 5'-TCTGGGAACTCGCTTTGTCTCCAA-3′ and 5′-GCTGGTTTGCTTTACAGT-CGCAGT-3′ for TGFβ1, and 5′-GGAGGGTGTTTGGGATCATTTTGG-3′ and 5′-GTCTCAGCACACTTCCATCTTGTT-3′ for TNFα. Relative mRNA expression levels were corrected for efficiency of the reaction using the Pfaffl method [19] and β_2-microglobulin and succinate dehydrogenase were used as internal reference standards. qPCR data are reported as means ± standard error of the mean (s.e.m.) and tested for statistical significance using one-way ANOVA followed by Tukey's post hoc test (IBM SPSS version 19).

BLAST analysis

NCBI protein BLAST searches were performed between reference proteins using default values and human (taxid:9606) and *Danio rerio* (taxid:7955) sequences. When a zebrafish homolog of the human signature gene was readily identifiable by gene name, a BLAST was run using the zebrafish protein as the query and the human reference protein sequences to determine the reported level of similarity

Please cite this article as: T.M. McDonald, et al., Zebrafish keratocyte explant cultures as a wound healing model system: Differential gene expression & morphological changes support epithelial–mesenchymal transition, Exp Cell Res (2013), http://dx.doi.org/10.1016/j.yexcr.2013.03.036