

RESEARCH ARTICLE

Dual inhibition of TGF β R and ROCK reverses the epithelial to mesenchymal transition in collectively migrating zebrafish keratocytes

Agnes S. Pascual¹ | Jose L. Rapanan² | Chandana K. Uppalapati³ |
Kimbal E. Cooper¹ | Kathryn J. Leyva³  | Elizabeth E. Hull¹ 

¹Biomedical Sciences Program, College of Graduate Studies, Midwestern University, Glendale, Arizona, USA

²Arizona College of Osteopathic Medicine, Midwestern University, Glendale, Arizona, USA

³Department of Microbiology & Immunology, College of Graduate Studies, Midwestern University, Glendale, Arizona, USA

Correspondence

Elizabeth E. Hull, Biomedical Sciences Program, College of Graduate Studies, Midwestern University, 19555 N. 59th Avenue, Glendale, Arizona, 85308 USA.
Email: ehullx@midwestern.edu

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Abstract

There is a growing controversy about the role of the epithelial to mesenchymal transition (EMT) in the fibrosis associated with chronic disease. Recent studies suggest that it is not the EMT transcriptional program but differentiation of progenitor cells, response to chronic inflammation, or some combination of both which cause the appearance of fibroblasts and the production of the extracellular matrix. To address this issue, we study the EMT process in the zebrafish keratocytes which migrate from primary explants of epithelial tissue as these cells are both terminally differentiated and able to divide. To firmly place this EMT process in the context of other systems, we first demonstrate that the zebrafish keratocyte EMT process involves nuclear accumulation of twist and snail/slug transcription factors as part of a TGF β R-mediated EMT process. As assessed by the expression and localization of EMT transcription factors, the zebrafish keratocyte EMT process is reversed by the addition of Rho-activated kinase (ROCK) in combination with TGF β R inhibitors. The complete cycle of EMT to MET observed in this system links these in vitro results more closely to the process of wound healing in vivo. However, the absence of observable activation of EMT transcription factors when keratocytes are cultured on compliant substrata in a TGF β 1-containing medium suggests that ROCK signaling, initiated by tension within the sheet, is an essential contributor to the EMT process. Most importantly, the requirement for ROCK activation by culturing on noncompliant substrata suggests that EMT in these terminally differentiated cells would not occur in vivo.

KEYWORDS

actin, protein kinases/phosphatases, protein signaling modules/scaffolds, transcription/transcription factors

Agnes S. Pascual and Jose L. Rapanan are co-first authors.

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1 | INTRODUCTION

Fibrosis is a major contributor to the pathology of chronic diseases. While the deposition of connective tissue in response to damage may restore the structural integrity of the tissue, the progressive replacement of parenchyma with fibrotic tissue eventually impairs organ function. EMT (epithelial to mesenchymal transition) has long been viewed as a key contributor to fibrosis. This transcriptional program in which epithelial cells gain mesenchymal characteristics similar to fibroblasts plays a key role in embryogenesis and cancer metastasis (type 1 and 3 EMT, respectively). In wound healing or tissue repair associated with chronic diseases, the apparent conversion of epithelial cells into fibroblasts or mesenchymal cells has been referred to as type 2 EMT. Each type of EMT involves the engagement of TGF β signaling and a set of transcription factors with overlapping functions including twist, snail/slug, and β -catenin-TCR/LEF (Kalluri & Weinberg, 2009).

However, a number of recent articles question the relevance of type 2 EMT to the fibrotic processes associated with chronic disease (Bartis et al., 2014; Fabris et al., 2016; Fragiadaki & Mason, 2011; Galichon et al., 2013; Kage & Borok, 2012; Kriz et al., 2011; Loeffler & Wolf, 2015; Xu & Dai, 2012). Much of the debate centers around the failure of several *in vivo* lineage tracing experiments to support a role for type 2 EMT (Fintha et al., 2019; Fragiadaki & Mason, 2011). Others suggest that inflammatory processes may contribute significantly to these fibrotic processes (Wynn & Ramalingam, 2012). In addition, some suggest that evidence supporting type 2 EMT *in vivo* arises from progenitor cells which may be more plastic than other cell types in the surrounding tissue (Fabris et al., 2016). A clearer understanding of the role of type 2 EMT may aid in the development of effective therapies targeting fibrosis which has been challenging to date (Fintha et al., 2019).

Several factors contribute to the controversy surrounding the physiological relevance of type 2 EMT. Much of the molecular characterization of EMT has been done utilizing cancer cell lines, frequently derived from carcinomas, and sometimes from metastases. While these may be effective tools for the delineation of the EMT transcriptional program, they are distinctly different from normal tissue. Specifically, when compared to normal tissues, the cells used in these experiments may have at least partially initiated an EMT process before the onset of experimental protocols. In addition, routine culture conditions potentiate the EMT process. Fetal bovine serum (FBS) supplementation exposes cells to TGF β and the rigid glass or plastic culture dish activates Rho-associated kinase (ROCK; Lessey et al., 2012). That said, these cell culture studies have generated a wealth of data that broadly agree with characterized EMT *in vivo*. In addition, established EMT is hypothesized to subsequently produce a mesenchymal to epithelial transition (MET). Indeed, the MET process is integral to development and wound healing, but EMT reversal is thought to be incomplete in fibrosis and in aggressive cancers. Many *in vitro* studies focus only on EMT and do not address MET as an integral part of the overall physiological process.

The unique aspects of the zebrafish keratocyte system have the potential to partially bridge this gap when considering the type of EMT seen in wound healing. First, this system does not rely on transformed cells and explants, at least initially, and maintains many aspects of the multicellular structure of skin tissue within the fish. Second, keratocytes migrate from the explant collectively in cell sheets in a manner that recapitulates much of the cell migration seen *in vivo* (Richardson et al., 2013). However, the fact that experiments are done *in vitro* allows detailed observation under a variety of controlled culture conditions and treatments (Rapanan et al., 2015).

Primary explant cultures of epithelial tissue are established by removing scales from an anesthetized zebrafish and culturing the tissue removed with the scale in a medium containing TGF β supplied by the FBS supplement (Grainger et al., 2000; Oida & Weiner, 2010). Thus, with the initiation of explant culture, an epithelial to mesenchymal transition (EMT) is initiated. Gene expression changes are detectable within hours after establishing the culture but disassembly of cell-cell junctions and fragmentation of the cell sheet occurs gradually over three to seven days (McDonald et al., 2013). After the establishment of cultures, expression of both TGF β ligand and TGF β receptors (RI and RII) increase over time (Tan et al., 2011). Our previous work in this system quantitated gene expression changes over time and identified transcription signatures for EMT, inflammation, and wound healing. In addition, the reorganization of the actin cytoskeleton from a meshwork in the lamella to stress-fibers in the more mesenchymal cells correlates with a shift from E-cadherin to N-cadherin expression previously shown to be characteristic of the EMT process in this system (McDonald et al., 2013).

Keratocytes collectively migrate from the explanted tissue concurrently with this EMT process. Sheets of keratocytes begin to collectively migrate from the scale within minutes. Within the sheet, leader cells with extensive lamellae, linked by a supracellular actin cable, function to pull the keratocyte sheet away from the scale. The formation of the supracellular actin cable appears to be dependent on the interaction of the phosphorylated myosin light chain with actin filaments which are anchored in cell-cell junctions at the leading edge (Morita et al., 2011; Rapanan et al., 2014). ROCK phosphorylates myosin light chains to increase stress fiber formation, which is evident in the supracellular actin cable coordinating migration at the leading edge of the sheet (Rapanan et al., 2014). When the formation of the cable is inhibited, forward migration is inhibited and cell-cell junctions appear to be dismantled. Inhibition of ROCK disrupts the supracellular actin cable and halts the forward motion of the sheet (Rapanan et al., 2014). In addition, when the lamellar extension is interrupted through inhibition of adhesion formation, the contractile force generated by the supracellular cable is no longer balanced and the cell sheet rapidly retracts (Rapanan et al., 2014). Thus, this experimental system allows for the study of both collective cell migration and the relationship between this process and signal transduction in EMT.

Data presented here extend the characterization of the EMT process initiated with explant culture. We focus on two of the factors that may contribute to EMT in other systems: TGF β signaling and

rigid substrata. We ask if the characterized gene expression changes downstream of TGF β signaling are associated with activation of transcription factors associated with EMT in mammalian systems and in vivo studies in zebrafish. Our previous work indicated that the downstream canonical targets such as changes in cadherin expression and reorganization of the actin cytoskeleton were similar to other EMT processes but did not address the activity of other EMT-associated transcription factors. In addition, we address the role of ROCK in this EMT process. TGF β 1 has been shown to promote EMT through ROCK activation (Bhowmick et al., 2001; Bhowmick et al., 2003; Cho & Yoo, 2007) and ROCK signaling plays a role in the dissolution of tight junctions during EMT (Zhang et al., 2013). Finally, as the ability to revert to an epithelial phenotype is the completion of the process, we also ask if dual inhibition of TGF β and ROCK signaling can lead to a MET process. Thus, in this study, we further characterize the EMT process, asking a series of questions including (1) Does TGF β signaling activate EMT-related transcription factors? (2) What roles do tension and the related activation of Rho-kinase play? and (3) Is the EMT process reversible in this system? More importantly, the results from this system using a cell type that is untransformed, terminally differentiated, and capable of cell division, have implications for the relevance of the EMT transcriptional program in fibrosis.

2 | MATERIALS & METHODS

2.1 | Explant culture and inhibitors

Keratocytes from anesthetized zebrafish were cultured using standard methods in DMEM with Hepes pH 7.4, 10% fetal bovine serum (FBS), 100 μ g/ml kanamycin, and 50 μ g/ml gentamicin (VWR) and grown at 28°C in 5% CO₂ (Rapanan et al., 2015). Cultures were established in glass-bottom culture dishes (Falcon) or 35 mm dishes with 28 kPa elastic supported surface compliant substrata (Ibidi). Cultures were treated with purified human TGF β (R&D Biosystems), 20 nM TGFRI inhibitor (ALK5 Inhibitor II or 2-(3-(6-Methylpyridin-2-yl)-1H-pyrazol-4-yl)-1,5-naphthyridine (Enzo)), LY2109761 (Selleck Chemicals), 20 μ M ROCK (Rho-associated protein kinase) inhibitor Y-27632 (Calbiochem).

2.2 | Immunofluorescent staining

Immunofluorescence microscopy using rabbit polyclonal antibodies characterized for human antigens (see table) and phalloidin (Invitrogen) was performed according to previous procedures (McDonald et al., 2013). Briefly, cultures were fixed with 4% paraformaldehyde, permeabilized, blocked, and incubated with primary rabbit polyclonal antibodies. Specific primary antibodies used were as follows: 1:1000 dilution of anti-snail/slug specific for pSer246 (Abcam ab63568), and a 1:500 dilution of anti-twist (Abcam ab49254) and anti- β -catenin (Y654, Abcam ab24925). Alexa Fluor

conjugated secondary antibodies were used (Invitrogen), after which cells were counterstained with phalloidin (Invitrogen) and mounted using Prolong Gold antifade containing DAPI (Invitrogen).

2.3 | Statistical analysis

Unpaired t-tests were used when comparing levels of a dichotomous independent variable (GraphPad Prism). All tests were two-tailed and the significance level was set at 0.05.

3 | RESULTS

3.1 | TGF β -mediated signaling activates EMT-related transcription factors

As TGF β R signaling plays a well-established and pivotal role in EMT (Valcourt et al., 2005; Zavadil et al., 2001), we started by asking if this cytokine activated the EMT-associated transcription factors snail/slug and twist (SNAI1, SNAI2, and TWST1). We were able to utilize a rabbit polyclonal to twist due to the relatively high sequence homology between the mammalian and zebrafish proteins (~70% identity). However, accurate localization for snail/slug proved technically challenging due to the limited homology between mammalian and zebrafish sequences (51% and 56% identity, respectively). By utilizing a polyclonal antibody raised to a peptide surrounding pSer246, a highly conserved region surrounding a posttranslational modification catalyzed by PAK1 involved in nuclear localization and gene expression (Yang et al., 2005), a reproducible immunofluorescence staining pattern emerged. As shown in Figure 1, there is apparent upregulation and increased nuclear localization of snail/slug and twist, suggestive of downstream, functional activation of these transcription factors. The nuclear localization of these transcription factors is reduced when TGF β R inhibitors are added to the culture medium (Figure 1). Although expression and nuclear localization are reduced with the addition of TGF β R inhibitors, the fragmentation of the cell sheet, which occurs in untreated cultures and is characteristic of this system (McDonald, 2013), appears to be unaffected. These data support the nuclear localization of the snail/slug and twist transcription factors downstream of TGF β R signaling.

We focused next on the ability of TGF β R inhibition to reverse an EMT or to initiate a MET process. As shown in Figure 2, we investigated the ability of TGF β RI inhibitor to reverse the EMT process as measured by nuclear localization of twist after EMT initiation. Addition of TGF β RI inhibitor after 48 h of explant culture, at a point when EMT transcription factors levels are elevated and localized in the nucleus, leads to a reduction in nuclear localization of these transcription factors within 24 h and a reduction in both levels and nuclear localization within 48 h of treatment. However, the morphology of these cells appears to be mesenchymal with disassembled cell-cell junctions and actin filaments. Thus, we searched for additional tools to promote EMT reversal.

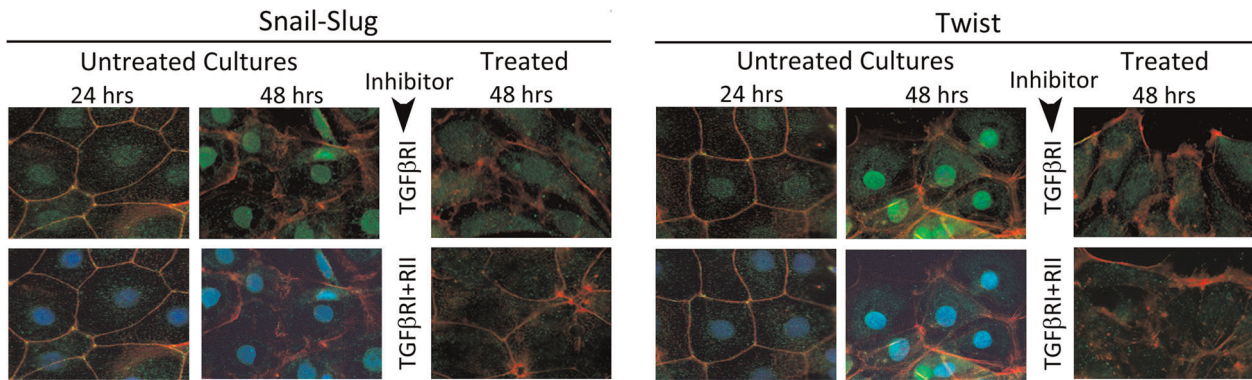


FIGURE 1 Levels and nuclear localization of snail/slug and twist appear to increase between 24 and 48 h of explant culture. Nuclear localization observed at 48 h is reduced when an inhibitor of TGF β R signaling is added at 24 h. The addition of TGF β RI inhibitor alone shows reduced localization while the addition of TGF β RI and RII inhibitors appears to decrease both expression levels and transcription factor nuclear localization. Key: transcription factors (green), actin (red), nucleus (blue)

3.2 | Contribution of tension to activation of rho-associated kinase

As Rho-activated kinase (ROCK) inhibition has been shown to lead to a mesenchymal to epithelial transition (MET) in the context of TGF β R mediated EMT (Das et al., 2009; Morin et al., 2011), we asked if inhibition of ROCK might promote the MET process. To do this, we utilized the ROCK inhibitor Y-27632, which is an ATP-competitive pyridine derivative (Uehata et al., 1997). As shown in Figure 2, the addition of this ROCK inhibitor with TGF β RI inhibitor 48 h after the establishment of explant cultures led to a more rapid decrease in the nuclear localization and levels of twist over TGF β RI inhibitor alone. In addition, there appears to be less fragmentation of the cell sheet. Given the previously characterized disruption in sheet migration with ROCK inhibition (Rapanan et al., 2014), we next utilized a less disruptive way to inhibit ROCK activity.

As shown in Figure 3, culture on compliant substrata leads to a reduction in cell area and internuclear distance. Specifically, after 24 h of culture on compliant substrata, these measures of cell spreading are equivalent to values obtained after 4 h of culture on glass. Less cell and sheet spreading is consistent with less tension and, under these conditions, there appear to be low and diffuse levels of twist staining with limited nuclear localization. When cultured on glass, twist levels are higher and localized within the nucleus where the visualization by fluorescence affects the observed color of the DAPI counterstained nuclei. Given the decrease in staining intensity and the documented links between ROCK and TGF β signaling, we asked if additional TGF β would increase the observed intensity of staining. The addition of 100 nM TGF β to the cultures does not appear to increase levels of twist, implying that additional TGF β is unable to counter the effects of culture on compliant substrata.

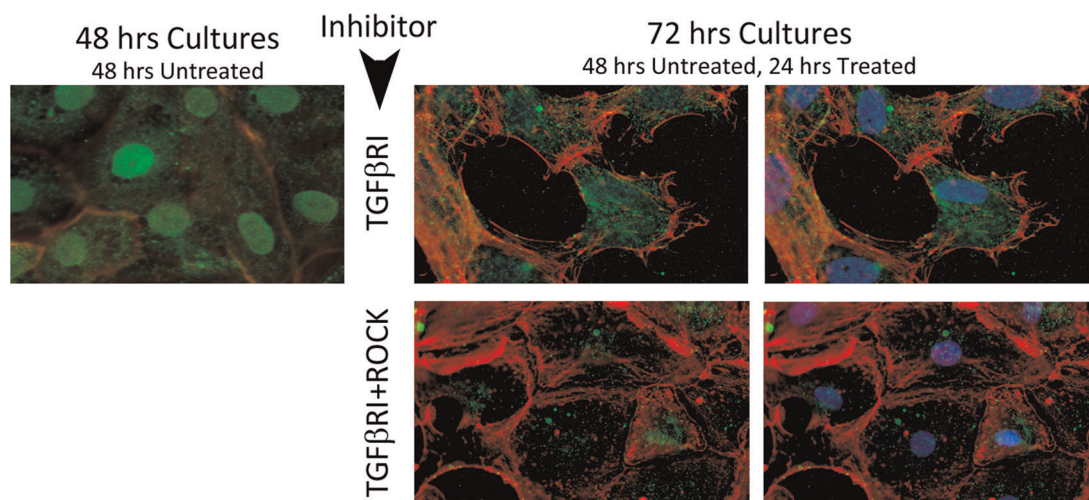


FIGURE 2 Addition of TGF β RI inhibitor 48 h after cultures were initiated leads to a redistribution of twist within 24 h (total culture time of 72 h). The combined treatment with ROCK inhibitor plus TGF β RI inhibitor, added 48 h after cultures were initiated, further reduces staining intensity of twist. Key: transcription factors (green), actin (red), nucleus (blue)

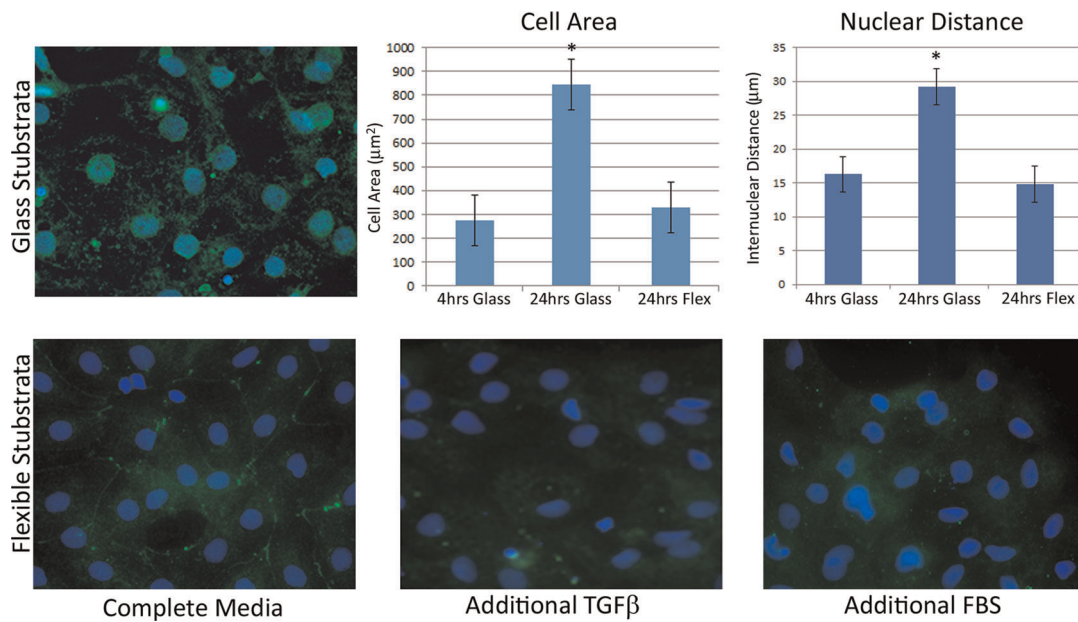


FIGURE 3 After culture of explants for 24 h on glass, staining with anti-twist yields more intense staining with increased nuclear localization than seen when explants are cultured on flexible substrata (surface elasticity 28 kPa). Quantitation of the distance between nuclei and the cell area suggests that the cell sheet is less spread after 24 h of explant culture on compliant substrata than on glass ($p < .05$) and indistinguishable from values seen after 4 h of culture on glass. Key: transcription factors (green), nucleus (blue)

To further assess changes consistent with EMT on compliant substrata, we focused on the disassembly of cell–cell junctions through nuclear translocation of β -catenin. In the epithelial state, this protein co-localizes with E-cadherin at cell–cell junctions. During disassembly of cell–cell junctions during EMT, β -catenin translocates to the nucleus where it associates with RCF/LEF transcription factors to promote EMT. This translocation is promoted by phosphorylation of β -catenin on tyrosine 654 (van Veelen et al., 2011).

Utilizing an antibody that detects both phosphorylated β -catenin (Y654) and unphosphorylated band(s), there is noticeable β -catenin staining at the periphery of the cells, colocalizing with the cortical actin cytoskeleton, regardless of substratum compliance. However, on compliant substrata, there appears to be a notable lack of nuclear β -catenin (Figure 4, top left panel). To expand on this observation, we quantitated the β -catenin staining in areas counterstained with DAPI to mark the nucleus. There is a statistically significant decrease in the

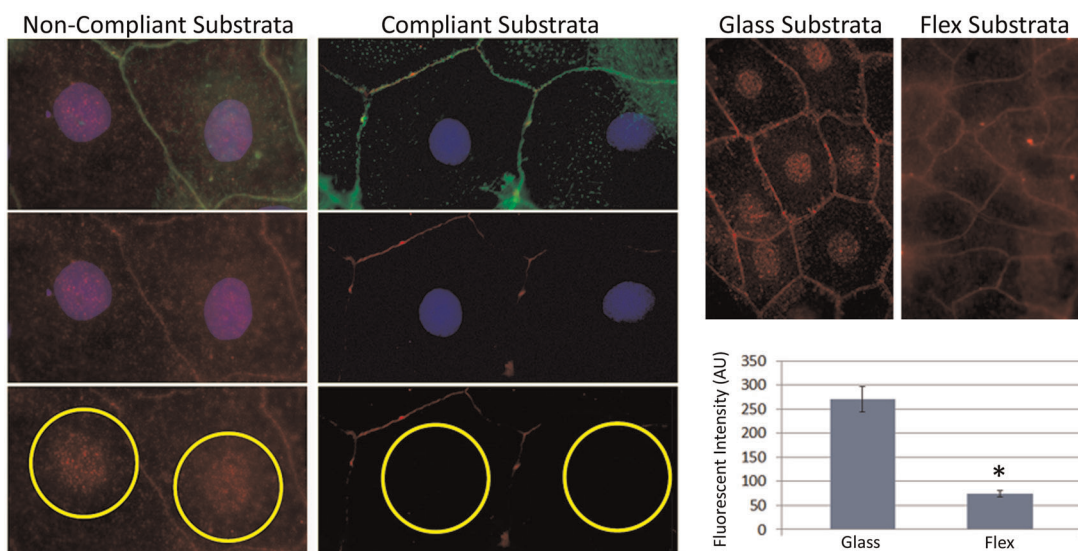


FIGURE 4 β -catenin levels are increased after culture of explants for 24 h on glass, with staining more intense both at the cell–cell junctions and in the nucleus when compared to cultures on compliant substrata (surface elasticity 28 kPa). Quantitation of fluorescent intensity of β -catenin staining with the culture is significant ($p < .05$). Key: β -catenin (red), actin (green), nucleus (blue)

level of β -catenin detected in the nucleus (Figure 4, bottom panels) and an observable decreased intensity observed in colocalizing with the cortical actin cytoskeleton (Figure 4, upper right panels). These data are consistent with reduced nuclear localization of an additional EMT-promoting transcription factor when cells are cultured on compliant substrata.

4 | DISCUSSION

Results presented here suggest that EMT in primary explant cultures of zebrafish keratocytes is dependent on TGF β R and ROCK signaling. Both signaling pathways are activated during the establishment of explant cultures as the stiffness of growth surfaces and TGF β concentrations markedly increase simultaneously. These pathways signal through the well-established EMT transcription factors snail, slug, and twist. In addition, the establishment of primary cultures on compliant substrata revealed a central role for ROCK signaling as ROCK appears to have a greater influence on EMT than TGF β during initiation of the process (Figure 3). Although inhibition of TGF β R signaling greatly reduces EMT as measured by transcription factor mobilization, reversal of EMT requires that both of these signal transduction pathways be inhibited.

At the molecular level, our findings are consistent with the accumulating evidence of crosstalk between TGF β and ROCK signaling in promoting EMT and the need for the inhibition of both pathways to induce MET. Thus, these data directly parallel results seen in many *in vitro* mammalian systems typically using cell lines cultured on noncompliant surfaces (Bhowmick et al., 2001; DasBecker, Hoffmann and Mertz, 2009; Kamaraju & Roberts, 2005; Korol et al., 2016; Xu & Dai, 2012). The reversal of an on-going EMT process is important as complete wound healing involves a mesenchymal to epithelial transition (MET) after EMT has successfully completed wound healing. As the EMT-MET process has been implicated in inappropriate healing and scarring (Haensel & Dai, 2018; Stone et al., 2016), a greater understanding of how the EMT process may be controlled during epithelial wound healing is needed.

Culture on compliant substrata, using surfaces closer to the stiffness of the ECM *in vivo*, was selected as a more physiological method to investigate the EMT process. The compliance of the substrata was chosen to mirror the *in vivo* environment of the keratocyte, between the rigidity of the scale and collagen dermis and approximately the stiffness of fibrotic tissue (Browning et al., 2013; Vernerey & Barthelat, 2010; Zhu et al., 2012). Not only are actin stress fibers more prominent when cells are cultured on non-compliant substrata (Tojkander et al., 2012), this altered morphology is linked to ROCK activity (Allen et al., 2012). Of particular interest to this study is the link between compliant substrata and TGF β signaling in differentiation pathways including EMT (Allen et al., 2012; Markowski et al., 2012). Given that latent TGF β may be activated by stiff ECM (Wells, 2013), culture on noncompliant substrata such as glass or plastic may be sufficient to initiate an EMT process. Indeed, preliminary data suggest that nuclear localization of twist is seen in

explant cultures established on glass surfaces in serum-free medium. However, it is unclear whether activation of latent TGF β or increased TGF β expression within the explant (which has been documented in this system (Tan et al., 2011) is supplying sufficient TGF β to initiate an EMT process in conjunction with the noncompliant substrata. In either case, these results are consistent with a model in which the same conditions that activate ROCK also activate TGF β R signaling to generate a robust EMT process.

The contribution of ROCK to the EMT-MET process is of particular interest given the role of this kinase in sheet migration. Despite similarities to EMT in mammalian systems at the molecular level which includes phosphorylation sites, previously published data document that the rate of keratocyte migration decreases during EMT. When explant cultures are initiated, there is an initial period in which tissue attaches to the substrata but, within 4 h, a collectively migrating cell sheet becomes apparent. In 24 h explant cultures, individual keratocytes migrate at speeds up to 0.1–0.5 microns per minute (Lee & Jacobson, 1997; Lee et al., 1993) and, when migrating collectively in sheets, the leading-edge advances at 25 microns per hour (Lee & Jacobson, 1997; Rapanan et al., 2014; Rapanan et al., 2015). This rapid rate, 10–100 fold more rapid than most cell types, is supported by labile close contact adhesions used by individually migrating keratocytes (Lee & Jacobson, 1997). However, over time in culture, the rate of keratocyte migration decreases as cells appear to lose their specialization for rapid motility, shifting from utilizing close contacts to adhesions more closely resembling focal adhesions (de Beus & Jacobson, 1998; Lee & Jacobson, 1997). As adhesion strength is inversely proportional to the speed of keratocyte migration (de Beus & Jacobson, 1998; DiMilla et al., 1991; Lee & Jacobson, 1997), it is significant that preliminary data suggest that adhesion strength increases over time in explant culture. Thus, the decrease in migration speed characteristic of EMT in this system is likely to be due to the increased adhesion strength.

Although this system offers unique insights into the EMT process, it does also have limitations. The establishment of explant cultures in a serum-free medium is problematic and, as serum contains substantial amounts of TGF β (Bandyopadhyay et al., 2006; Oida & Weiner, 2010; Tan et al., 2011), it is technically difficult to remove exogenous TGF β from the system. Specifically, we have observed that when explants are cultured in a serum-free medium, cell-cell junctions rapidly disassemble (data not shown) which effectively removes the supracellular actin cable required for the generation of tension within the sheet and subsequent ROCK activation. In addition, as EMT is well established in this system (McDonald et al., 2013), we have utilized expression and nuclear localization of EMT-related transcription factors as our primary assay for an EMT process. This rapid assay may not allow us to distinguish between certain aspects of the EMT process.

These data suggest that zebrafish keratocytes do not undergo an EMT process when cultured on a substratum that mirrors the compliance of their *in vivo* environment, even in the presence of high levels of TGF β . As in mammals, the EMT transcriptional program is activated in zebrafish embryogenesis (Fazilaty et al., 2019) and the

signaling pathways required for EMT are still present in terminally differentiated zebrafish keratocytes (McDonald et al., 2013) but maybe limited in vivo. It should be noted that our experimental approach utilized noncompliant substrata to mechanically activate ROCK and does not address the possibility that, in vivo, ROCK may be activated by another mechanism that might allow a type 2 EMT process to be initiated. Taken together, these findings support the growing view that type 2 EMT may be more limited than previously believed.

5 | CONCLUSION

Our data provide the support that initiation and maintenance of EMT vary depending on the environment of the cells, and are not as closely tied to TGF- β levels as previously believed. While TGF- β can induce a transcriptional EMT program both in vitro and in vivo, we observed a difference in the transcriptional profile in vitro that appears to be both dependent on the compliance of the cellular substrata and independent on the concentration of TGF- β . Our data may shed light on how the specific nature of a type 2 EMT program may be more limited in vivo than originally described.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Kathryn J. Leyva  <http://orcid.org/0000-0001-5093-6315>

Elizabeth E. Hull  <http://orcid.org/0000-0001-6100-9605>

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