Award Number: W81XWH-09-1-0497

TITLE: Hic-5's Regulatory Role in TGFB Signaling in Prostate Stroma

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REPORT DATE: July 2011

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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1. REPORT DATE  
July 2011

2. REPORT TYPE  
Annual Summary

3. DATES COVERED  
1 July 2010 – 30 June 2011

4. TITLE AND SUBTITLE  
Hic-5's Regulatory Role in TGFB Signaling in Prostate Stroma

5a. CONTRACT NUMBER  

5b. GRANT NUMBER  
W81XWH-09-1-0497

5c. PROGRAM ELEMENT NUMBER  

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)  
University of Pittsburgh  
Pittsburgh, PA

8. PERFORMING ORGANIZATION REPORT NUMBER  

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)  
U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)  

11. SPONSOR/MONITOR'S REPORT NUMBER(S)  

12. DISTRIBUTION / AVAILABILITY STATEMENT  
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES  

14. ABSTRACT  
None Provided

15. SUBJECT TERMS  

16. SECURITY CLASSIFICATION OF:  

a. REPORT  
U

b. ABSTRACT  
U

c. THIS PAGE  
U

17. LIMITATION OF ABSTRACT  
UU

18. NUMBER OF PAGES  
15

19a. NAME OF RESPONSIBLE PERSON  

19b. TELEPHONE NUMBER (include area code)  

# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>4</td>
</tr>
<tr>
<td>Body</td>
<td>4</td>
</tr>
<tr>
<td>Key Research Accomplishments</td>
<td>8</td>
</tr>
<tr>
<td>Reportable Outcomes</td>
<td>8</td>
</tr>
<tr>
<td>Conclusion</td>
<td>9</td>
</tr>
<tr>
<td>References</td>
<td>9</td>
</tr>
<tr>
<td>Appendices</td>
<td>10</td>
</tr>
</tbody>
</table>
INTRODUCTION:

In this study, we attempt to demonstrate that a reactive stroma maintains the ability to restrain cancer cell motility, but this inhibition is lost when local TGFβ production stimulates an increased production of stromal ROS that in turn inactivates the inhibitor. We show that in the absence of TGFβ, a myofibroblastic prostate stromal cell (WPMY-1) produces a motility inhibitory factor that limits motility in a highly aggressive PCa cell line (DU145). Additionally, we identified Cox-2 as the downstream target of TGFβ responsible for the loss of this inhibition, and we show that the inactivation of the inhibitor is via a ROS-dependent mechanism generated as a byproduct of arachidonic acid conversion by Cox-2. These results suggest that local TGFβ production in the cancer milieu promotes increased metastatic potential by causing oxidative inactivation of a secreted stromal-derived regulatory factor.

BODY (all referenced figures and legends are attached separately as an appendix):

TGFβ mediates the differential ability of reactive versus non-reactive human prostate stromal cell lines to limit PCa cell motility

We have used a modified scratch assay to examine the impact of stromal cell derived factors on PCa cell migration in vitro. This assay has been widely used and is well established to represent the classical “wound healing” migratory response in cells without the focus on chemotaxis seen with the use of Boyden chambers [1]. We modified this assay to investigate the migration activity of the androgen-independent DU145 human PCa cell line when co-cultured with different human prostate stromal cell lines. We chose the DU145 cell line as it is known to secrete high levels of active TGFβ, a known mediator of stromal cell transdifferentiation [2]. The WPMY-1 cell line provides a model of “reactive” stroma given their myofibroblast phenotype while the PS-30 are more fibroblastic and therefore more reflective of non-reactive stroma. Importantly, in the co-culture system that we employ the stromal and PCa cells are not in direct contact but share a common growth medium.

Figure 1 displays the results of a scratch assay with DU145 cells co-cultured with either WPMY-1 or PS30 cells. DU145 cells plated at ~90% confluence can close a constant diameter wound approximately 22% within 24hrs in low serum media. While the motility of these cells is not significantly affected when co-cultured with WPMY-1 cells, their movement is significantly reduced upon co-culture with PS30 cells. Since TGFβ is an important contributor to the reactive stromal phenotype in the prostate, we used an interfering TGFβ antibody to block its signaling in the co-culture system. As shown in Figure 1A, inhibition of TGFβ signaling did not affect the inherent motility of DU145 cells but uncovered a migration inhibitory activity in the WPMY-1 cells. The anti-motility activity of the PS30 cells was not affected by the TGFβ neutralizing antibody. These results suggest that while TGFβ does not affect DU145 cell movement, it is required for reactive stromal cells to obtain their permissive role in cancer cell motility.

Transient transfection of both the WPMY-1 and PS30 cell lines with 3TP-lux, a Smad binding element luciferase reporter construct, shows that the WPMY-1 line has a significantly more robust response to exogenous TGFβ than the PS30 line (data not shown). This differential response to TGFβ likely contributes to the loss of motility-limiting activity in the WPMY-1 line. Additionally, these data suggest that while both reactive and non-reactive prostate stromal cells produce an inherent cancer cell A

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migration inhibitory factor(s), reactive stroma respond to TGFβ produced by cancer cells to limit the production or activity of this factor(s). As a direct test of this hypothesis, we isolated conditioned media from WPMY-1 cells grown overnight in 1% serum-containing media with or without exogenous TGFβ (5 ng/mL) and added this media to freshly wounded naïve DU145 cells. Surprisingly, conditioned media from WPMY-1 cells treated with exogenous TGFβ significantly inhibited DU145 motility (Fig 1C). Therefore, either DU145 produce other factors that, in addition to TGFβ, are required to block the inherent migration inhibitory factor of WPMY-1 cells, or the effect of TGFβ on this inhibitor requires chronic local TGFβ production due to the involvement of a short-lived molecule.

**TGFβ induces a pro-oxidant and proinflammatory phenotype in reactive stromal cells**

TGFβ is an important growth factor in mediating stromal-epithelial cross-talk, and is necessary for the transdifferentiation into a myofibroblast and the development of a reactive stroma. The bio-assay we developed demonstrated a potent effect of a TGFβ-stimulated reactive stroma on the wound healing feature of the cancer cells, thus supporting a previously proposed theory of cancer as an overhealing wound [3]. Given this effect on wound healing activity, we sought to identify the TGFβ-dependent stromal factor(s) that limits the inherent migration inhibitory activity of reactive stroma. Since the inhibition effect seems to be short-acting (non-transferrable in conditioned media), we focused initially on local, short-acting mediators of inflammation and wound healing. ROS are trademark components of a healing wound and an inflammatory milieu, and are short-acting, non-transferrable mediators. Additionally, TGFβ is known to transcriptionally regulate several pro-oxidant and proinflammatory genes [4, 5].

To test our hypothesis that locally produced TGFβ is affecting a reactive stroma via increased generation of ROS, we performed an Amplex Red endpoint assay measuring H2O2 production by TGFβ stimulated WPMY-1 cells. As Figure 2a shows, a 3h TGFβ treatment significantly increases production of H2O2 at both 2 and 5 ng/mL doses. This is consistent with previous reports that TGFβ upregulates enzymes involved in the production of H2O2 [4].

To confirm that TGFβ treatment is upregulating a key enzyme involved in ROS generation and inflammation in our system, we indirectly co-cultured WPMY-1 cells with DU145 cells using a transwell system. To replicate the conditions in which we observed the change from a permissive to inhibitive role of the WPMY-1 cells on DU145 motility, we used media containing either a control antibody or a TGFβ neutralizing antibody. We focused on a well-studied enzyme involved in wound healing and the inflammatory response, Cox-2. Subsequent analysis of the WPMY-1 cells by quantitative real-time PCR showed that, under control conditions, co-culture with DU145 cells increased mRNA transcript levels of Cox-2, consistent with the promotion of a pro-oxidant and pro-inflammatory milieu. This increase was blocked by the addition of a TGFβ neutralizing antibody (Fig 2b).

H2O2 is both short-acting and unstable, making it a viable candidate for the non-transferrable motility-permissive effect we see in DU145/WPMY-1 co-culture. To test this hypothesis, we repeated our modified wound healing assay with the addition of catalase (1500 units/mL). As Fig 2c shows, addition of catalase does not alter the inherent highly motile phenotype of the DU145 cells, but when added to the

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1. Cox, migration from modified permissive neutralizing catalase in inflammatory milieu.
2. TGFβ induces a pro-oxidant and proinflammatory phenotype in reactive stromal cells.
3. ROS, the local, short, and non-transferrable factors are involved in wound healing.
4. TGFβ is known to transcriptionally regulate several pro-oxidant and proinflammatory genes.
5. H2O2 production by TGFβ stimulated WPMY-1 cells is significantly increased at 3h.
6. Cox-2 transcript levels are increased under control conditions with DU145 cells.
7. Catalase addition does not alter the DU145 cells' phenotype.
co-culture system it reverses the permissive effect of the WPMY-1 cells and restores their migration inhibitory activity. This suggests that extracellular H$_2$O$_2$ is necessary for the permissive effect of WPMY-1 cells on DU145 motility, but that it is not necessary for inherent DU145 motility.

**Stromal Cox-2 is a significant source of ROS and is necessary for cancer cell motility.**

Conflicting evidence currently exists with the utility of using NSAIDs to reduce prostate cancer risk. Overall, NSAID usage is thought to have a small reduction in disease development risk [6], but the inadequacy of definitive results with such large scale trials underscores the need for a better understanding of the molecular role of Cox-2 in prostate cancer. With the wide array of FDA-approved Cox-2 inhibitors already available for use, we hypothesized that a better understanding of the molecular mechanisms of Cox-2’s involvement in PCa could potentially lead to greater therapeutic efficacy through combination therapies including a clinically available Cox-2 inhibitor. To determine to what extent stromal-derived Cox-2 is involved in cancer cell motility, we first generated a stable WPMY-1-derived variant that lacks TGFβ-mediated Cox-2 induction. Using a lentiviral vector expressing an shRNA sequence specific for human Cox-2 (SH4) or a scrambled shRNA sequence (Scr), we infected wild-type WPMY-1 cells and created a stably expressing SH4 line. Basal levels of Cox-2 are very low in WPMY-1 cells, so to test the efficiency of our knockdown we stimulated either wildtype WPMY-1 or SH4 cells with 5 ng/mL of TGFβ and subsequently analyzed Cox-2 mRNA transcript levels via qRT-PCR. As Figure 3a shows, our SH4 line demonstrates approximately 65% knockdown of Cox-2 induction in response to exogenous TGFβ.

As Fig 2c shows extracellular H$_2$O$_2$ is necessary for the alleviation of motility inhibition by the stroma, we sought to determine if the pharmacological target Cox-2 plays a role in the generation of H$_2$O$_2$. Previous work has shown that Cox-2 can be a significant source of ROS generation [7], so to test if Cox-2 can generate H$_2$O$_2$ in our system we subjected the SH4 cells to an endpoint Amplex Red assay identical to the one performed on WPMY-1 cells in Fig 2a. As Fig 3b shows, TGFβ is unable to produce a significant increase in H$_2$O$_2$ production in stromal cells lacking inducible Cox-2, suggesting that Cox-2 is at least partially responsible for the TGFβ induced increase in H$_2$O$_2$ production in WPMY-1 stromal cells.

Next, we sought to determine if ablation of Cox-2 and therefore decreasing the amount of H$_2$O$_2$ produced in response to TGFβ had a biological effect on the motility of DU145 cancer cells. Using our previously described modified wound healing assay, we show in Figure 3c that SH4 stromal cells retain their ability to inhibit cancer cell motility in a co-culture system when compared to their inducible Cox-2 counterparts, WPMY-1 and Scr lines. To further support our hypothesis that stromal Cox-2 is a significant source of H$_2$O$_2$ in our system, we repeated the assay with the addition of catalase (1500 units/mL). As expected, addition of catalase does not further accentuate the inhibitory activity of the stroma on cancer cell motility, indicating that Cox-2 is producing sufficient H$_2$O$_2$ to account for the effects seen by neutralizing H$_2$O$_2$ in the wild-type system (Fig 2c).

**Hydrogen peroxide produced by stromal Cox-2 modulates the activity of the inhibitor via oxidative stress signaling**

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In the absence of chronic TGFβ stimulation in co-culture with DU145 cells, the WPMY-1 line is able to produce an inhibitor of cancer cell motility. This inhibitor is transferrable in conditioned media, and is present in co-culture with DU145 cells when Cox-2 is ablated in the WPMY-1 line through a stable shRNA lentiviral transduction. We hypothesized that stromal Cox-2 was generating H2O2, and this was in turn modulating the effect of the inhibitor. To test this hypothesis, we generated conditioned media from WPMY-1 cells as previously described, and added varying concentrations of H2O2 to the media. We then used this media on naïve DU145 cells and performed the modified wound healing assay. As Figure 4a shows, a single bolus of H2O2 (5, 10 and 20 µM) alone does not significantly influence DU145 motility, but when added to the conditioned media the inhibition of motility is lost. This suggests either that the inhibitor that is transferrable in the conditioned media is being oxidatively modified by H2O2, or that the production of H2O2 by the stroma is acting as a signaling molecule and is influencing the response of the DU145 cells to the constitutively produced inhibitor. To further confirm that the restoration of the stroma’s inhibitory activity in the SH4 co-culture is due to a loss of H2O2 production and not a different Cox-2 metabolite, we repeated the DU145/SH4 wound healing assay with exogenous H2O2 added (10 µM). As expected, this reverses the inhibitory effect of the SH4 cells on DU145 motility (Figure 4b). When the major prostatic Cox-2 metabolite PGE2 was added back to the co-culture system, no significant change in motility was observed (data not shown).

To tease out the role of H2O2 more specifically, we designed an experiment to distinguish between direct oxidation of the inhibitor and oxidative stress signaling in the DU145 cells influencing the action of the inhibitor. We pre-treated conditioned media with 10 µM H2O2 for 3h to allow potential oxidation of the inhibitor. Prior to treating wounded naïve DU145 cells with the conditioned media, we added catalase (1500 units/mL) to neutralize the remaining H2O2. As Figure 4c shows, pre-treatment of the conditioned media does not alter its inhibitory effect, but addition of H2O2 to the DU145/conditioned media system does reverse the motility blockade. This suggests that the inhibitor itself is not being oxidized, but rather that H2O2 is acting directly on the DU145 cells’ response to the stromal-produced inhibitor.

**The androgen derivative 3β-Adiol is responsible for the inhibition of DU145 motility seen in co-culture with prostate stromal cells**

While DU145 cells are AR negative, they maintain the normal intracellular enzymatic machinery for steroid biosynthesis and steroid metabolism [8]. We thus postulated that the stromal-derived inhibitor of motility could potentially be a steroid or a steroid metabolite. We used a column fractionation technique to separate the components in the conditioned media into high (>5 kD) and low (<5 kD) molecular weight fractions, and used the separate fractions in our modified wound healing assay on naïve DU145 cells. As Figure 5a shows, the low molecular weight fraction retains the inhibitory activity on DU145 cell motility. The high molecular weight fraction is permissive of DU145 motility, and re-addition of the low MW fraction again restores the motility suppression seen in the standard conditioned media control.

Following a thorough literature search for potential low molecular weight inhibitors of prostate cancer cell motility, we hypothesized that an androgen derivative was responsible for the inhibition of motility seen in the absence of local TGFβ. Two androgen derivatives, 5α-androstane-3α,17β-adiol (3α-Adiol) A A A A
and 5α-androstane-3β,17β-adiol (3β-Adiol), do not bind AR but are potent ligands for ERβ [9]. Both of these metabolites increase cancer cell adhesion and decrease cell motility in an ERβ-dependent manner [9, 10]. DU145 cells are known to express the ERβ isoform but no detectable ERα [9-11]. To determine if this pathway was responsible for our oxidant-dependent inhibition of motility in co-culture, we used exogenous 3β-Adiol (10⁻⁶ M) in our modified wound healing assay with and without the addition of H₂O₂ (10μM). As previously reported, 3β-Adiol suppresses DU145 cell motility, but interestingly this effect is reversed with the addition of H₂O₂ (Figure 5b). To further support our hypothesis that our conditioned media contained an androgen metabolite capable of inhibiting cancer cell motility, we repeated the wound healing assay using conditioned media with the addition of the SERM tamoxifen, which acts as an ERβ antagonist, and the selective ERβ antagonist 4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol (PHTPP). As Figure 5c shows, addition of both tamoxifen and PHTPP reverses the motility suppression normally seen with the conditioned media. This indicates that the stromal produced inhibitor is acting through an ERβ-dependent mechanism.

**KEY RESEARCH ACCOMPLISHMENTS:**

- Evidence that a reactive stroma maintains the ability to produce an inhibitor of cancer cell motility, as an inhibitory activity is transferrable in stromal conditioned media
- Addition of exogenous H₂O₂ to simulate the redox status of an inflammatory milieu reverses the action of the stromal-derived inhibitor
- Cox-2 plays an important role in the cancer microenvironment and its TGFβ dependent induction is necessary for cancer cell motility; Stromal Cox-2 mRNA is directly upregulated in response to co-culture with PCa cells, and lentiviral-mediated knockdown blocks the TGFβ dependent reversal of motility inhibition
- The androgen derivative 3β-Adiol may be the stromal-produced inhibitor responsible for PCa motility inhibition. This metabolite signals through an ERβ dependent mechanism, and addition of H₂O₂ to the system inhibits its activity by acting directly on the cancer cell’s response to the ligand

**REPORTABLE OUTCOMES:**

- Manuscript in preparation reporting the current findings described above
- This work was presented at the AACR Conference on Metastasis and the Tumor Microenvironment in poster format held in Philadelphia Sept 12-15 2010
- Working model of our system has been developed (see schematic below); this provides the potential to identify druggable targets to prevent cancer cell metastasis
CONCLUSION:

Stromal expression of Cox-2 in response to local TGFβ production produces an increased concentration of ROS that can inhibit the action of a secreted inhibitor of motility, thus allowing aggressive cancer cells to migrate despite the attempt of the stroma to keep the system in check. The androgen derivative 3β-Adiol may be the inhibitor produced by the stroma. This compound signals through an ERβ-dependent pathway, and the increased production of ROS in the milieu may directly affect ERβ signaling in the cancer cells, thus influencing the effect of 3β-Adiol. Targeting this pathway may provide a novel approach to restraining cancer motility by using the inherent properties of the prostate stroma to limit cancer cell movement.

REFERENCES:


**APPENDICES:**

Figures and legends are attached (referenced in the Body section above)

**SUPPORTING DATA:**

See appendix
CAFs maintain ability to inhibit DU145 cell motility in the absence of active TGFB

Conditioned Media from WPMY-1 cells inhibits DU145 motility irregardless of TGFB treatment

Figure 1. A role for the stromal microenvironment in modulating cancer cell motility via paracrine interactions.

(A) In an indirect co-culture system, WPMY-1 cells are permissive of the highly motile phenotype of DU145 PCA cells, which are high secretors of active TGFβ. PS30 cells inhibit DU145 motility in co-culture. Addition of a TGFβ-neutralizing antibody blocks this motility allowance from the WPMY-1 cells in co-culture, but does not affect motility when added to DU145 cells alone or in co-culture with PS30 cells. The left panel is representative images from the modified wound healing assay. Data represent the mean ± SEM from 4 independent experiments, each repeated in technical triplicate. A one-way ANOVA followed by Tukey’s multiple comparison test was performed.*p<.05 compared to respective DU145 control. ** p<.01 relative to DU145 treated with TGFβ neutralizing antibody.

(B) This inhibition of migration is transferrable in conditioned media made from WPMY-1 cells grown overnight in 1% serum media -/+ TGFβ (5 ng/mL). Naive DU145 cells were wounded and the media was replaced with WPMY-1 conditioned media and the wound closure over 24h was determined. Data represent the mean ± SEM from 3 independent experiments, each repeated in technical triplicate. A one-way ANOVA followed by Tukey’s multiple comparison test was performed.** p<.01 compared to control media.
**Figure 2. CAFs produce a pro-oxidant and proinflammatory response to locally produced TGFβ.**

(A) In response to exogenous TGFβ, WPMY-1 cells produce increased levels of H₂O₂ as measured by an endpoint Amplex Red assay. WPMY-1 cells were serum starved for 90 min before addition of TGFβ in fresh serum free media. Cells were incubated with the TGFβ for 3h, Amplex Red was added to the system, and an endpoint reading was recorded at 1h. Data represent mean from 3 biological replicates ± SEM. A one-way ANOVA followed by Tukey’s multiple comparison test was performed.* p<.05, ** p<.01 relative to untreated WPMY-1 control.

(B) Co-culture with DU145 cells increases mRNA transcript levels of Cox-2 in WPMY-1 cells. A transwell insert containing DU145 cells was placed into a chamber containing serum-starved WPMY-1 cells, and the media was replaced with fresh 1% serum-containing RPMI -/+ a TGFβ neutralizing antibody. Control cells were incubated with an empty insert. The cells were co-cultured for 24h and the WPMY-1 cells from the lower chamber were collected in Trizol and subject to reverse transcription and quantitative real-time PCR for Cox-2 transcript levels. Data represent the mean ± SEM from 3 independent experiments. A one-way ANOVA followed by Tukey’s multiple comparison test was performed.* p < .05 relative to control and TGFβ neutralizing antibody.

(C) Addition of catalase to the co-culture system reverses the permissive role of WPMY-1 cells in DU145 motility. The modified wound healing assay was performed with the addition of 1500 units of catalase per 1 mL of media. Data are representative of 3 independent experiments ± SEM. A one-way ANOVA followed by Tukey’s multiple comparison test was performed.* p< .05 relative all other conditions.
Lentiviral shRNA Knockdown of Cox-2 in WPMY-1 PrStr Line Blocks TGFB dependent mRNA induction

WPMY-1 SH4 0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5
Fold change over control

TGFB treatment does not increase H2O2 production in SH4 cells

0 2 5 10
0 500 1000 1500
ng/mL TGFB

Figure 3. TGFB-inducible stromal Cox-2 is necessary for cancer cell motility through production of increased levels of H2O2

(A) A lentiviral vector expressing shRNA either scrambled (Scr) or directed against Cox-2 (SH4) was used to stably infect WPMY-1 cells. Following selection through puromycin and clonal expansion, the resulting lines were subjected to a 24h treatment with TGFB in serum-free media (5 ng/mL) and Cox-2 mRNA levels were determined by quantitative RT-PCR. Fold change over untreated controls was determined. Data represent 3 independent experiments ± SEM. A one-way ANOVA followed by Tukey’s multiple comparison test was performed. *p<.05

(B) In response to exogenous TGFB, SH4 cells lack an increase in H2O2 production as measured by an endpoint Amplex Red assay. SH4 cells were serum starved for 90 min before addition of TGFB in fresh serum free media. Cells were incubated with the TGFB for 3h, Amplex Red was added to the system, and an endpoint reading was recorded at 1h. Data represent mean from 3 biological replicates ± SEM.

(C) Addition of catalase to the co-culture system reverses the permissive role of Scr cells in DU145 motility but does not further accentuate the motility inhibition offered by the SH4 cells. The modified wound healing assay was performed with the addition of 1500 units of catalase per 1 mL of media. A one-way ANOVA followed by Tukey’s multiple comparison test was performed. * p<.05 relative to DU145 control.
Addition of H2O2 to Conditioned Media Reverses the Inhibitory Effect on DU145 Motility

**Figure 4. The stromal produced inhibitor can be inactivated via oxidation by H2O2**

(A) Conditioned media loses its inhibitory effect on DU145 motility at concentrations of H2O2 ≥ 10 μM. Conditioned media was generated as previously described from WPMY-1 cells. Naive DU145 cells were wounded and the media was replaced with conditioned media to which varying amounts of H2O2 had been added. Data represent the mean of 6 independent experiments ± SEM. A one-way ANOVA followed by Tukey’s multiple comparison test was performed. **p<.01 relative to appropriate control media

(B) Addition of H2O2 (10 μM) to the DU145/SH4 co-culture reverses the motility inhibition seen under basal conditions. A modified wound healing assay as previously described was carried out with and without the addition of H2O2. Data represent the results of 4 independent experiments ± SEM. A one-way ANOVA followed by Tukey’s multiple comparison test was performed. * p<.05 relative to appropriate DU145 control

(C) Conditioned media was incubated with 10 μM H2O2 for 3h. Following this pretreatment, a portion of the media was then treated with catalase (1500 units/mL) and the 2 different treatment groups were added to wounded naive DU145 cells. Data represent 3 independent experiments ± SEM. A one-way ANOVA followed by Tukey’s multiple comparison test was performed. * p<.05 compared to all other conditions
The stromal produced inhibitor is present in the low molecular weight fraction of conditioned media. DU145 control CM control >5K fraction <5K fraction combined 0 10 20 30% wound closure (24h)

Figure 5. 3β-Adiol from stromal conditioned media blocks DU145 motility in an ERβ-dependent manner

(A) Stromal conditioned media was fractionated into high and low molecular weight fractions, and each fraction was tested independently for biological activity. The low molecular weight fraction (pore size <5kD) retains inhibitory action on DU145 motility. Data are representative of 4 independent biological replicates ± SEM. A one-way ANOVA followed by Tukey’s multiple comparison test was performed, ***p<.001 compared to both DU145 control and the high molecular weight fraction.

(B) Addition of exogenous 3β-Adiol (10^-6 M) is able to significantly inhibit DU145 motility. This effect is reversed when 10 µM H2O2 is added to the media. Data are representative of 4 independent biological replicates ± SEM. A one-way ANOVA followed by Tukey’s multiple comparison test was performed. **p<.01 compared to all other conditions.

(C) Naïve DU145 cells were wounded and treated with either control or stromal conditioned media with 4-hydroxytamoxifen (10^-7 M) or PHTPP (0.1 µM). Results are representative of 4 independent biological replicates ± SEM. A one-way ANOVA followed by Tukey’s multiple comparison test was performed. ***p<.001 compared to conditions containing control media. φp<.05 compared to conditioned media containing conditions.