

Investigating the molecular mechanisms that govern stemness versus differentiation in salivary mucoepidermoid carcinoma

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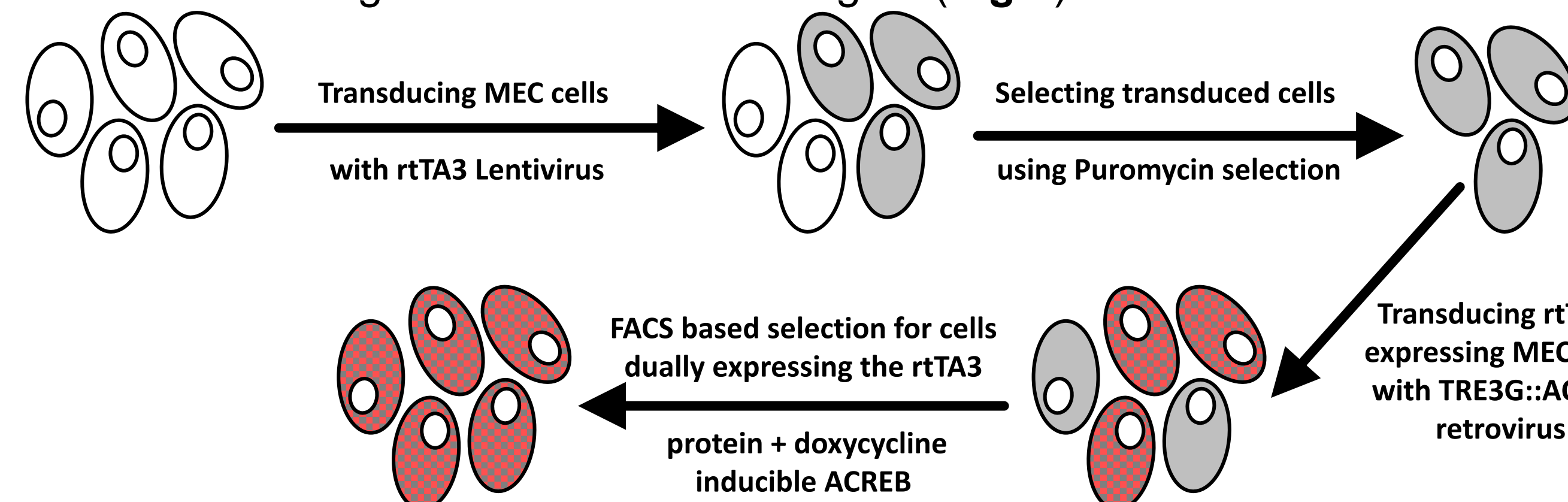
ABSTRACT

Mucoepidermoid carcinoma (MEC) is the most common salivary gland malignancy and is primarily driven (up to 80% of all cases) by a fusion oncogene. This oncogenic gene fusion arises from a chromosomal translocation event creating an in-frame fusion of CRTC1 and MAML2 genes (C1/M2). The majority of salivary MEC patients present with slow-growing, highly differentiated tumors (low-grade) and exhibit promising prognosis (90% 5yr-survival). However, some patients present with aggressive, poorly differentiated tumors (high-grade) that frequently recur and metastasize leading to dismal outcomes (25% 5yr-survival). We and others have shown that C1/M2 exerts its oncogenic abilities by acting as a constitutive co-activator for the 'master' transcription factor CREB, and a novel and previously unappreciated ability of C1/M2 to interact with another 'master' transcription factor and proto-oncogene MYC. Our recent work suggests that these interactions govern opposing functions of the CRTC1-MAML2 oncogene that regulate salivary MEC differentiation and stemness, respectively. However, their functional contribution towards MEC tumor biology, specifically in the low→high grade tumor transition, still remains entirely unknown. In this study, we found that expression of a dominant negative CREB molecule (ACREB) which effectively blunts C1/M2-CREB interactions and ablate overall CREB function, causes a dramatic change in cell morphology and behavior. Specifically, blunting CREB function in MEC cells leads to epithelial-mesenchymal transition (EMT), whereby cells exhibit increased single cell mobility, collective migration, and invasion which are all hallmarks of aggressive, poorly differentiated tumors (high grade) tumors. Thus, we have uncovered a novel and previously unreported role of CREB towards regulating salivary MEC tumor progression.

METHODS

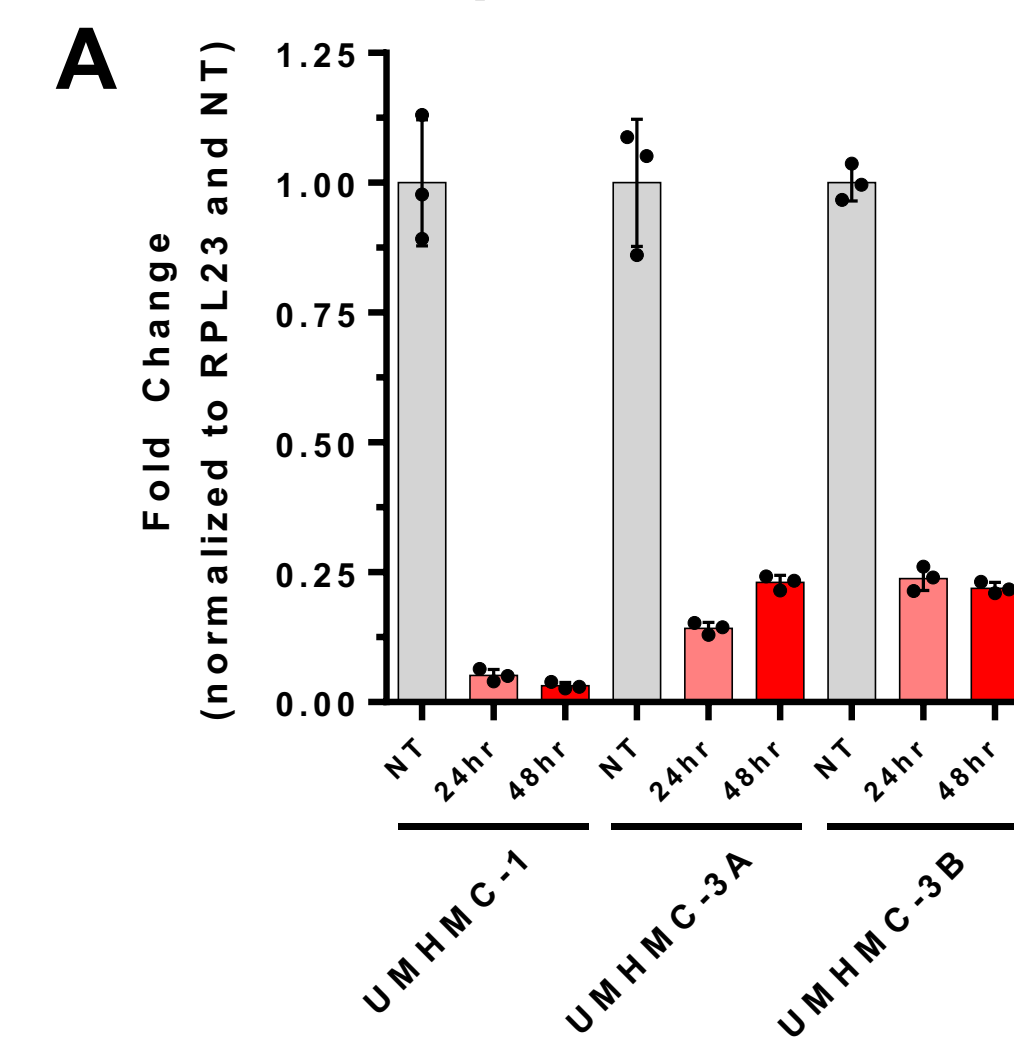
Generating stable mucoepidermoid carcinoma cell lines expressing doxycycline induced ACREB. MEC cells (UMHMC-1/3A/3B) were first transduced with a lentivirus containing a DNA cassette for the constitutive expression of the doxycycline-inducible reverse tetracycline transactivator 3 protein (rtTA3). This DNA cassette also contained a Puromycin resistance gene, which enabled us to select cells that were positively transduced with the lentivirus by selectively killing off negative cells via puromycin treatment. rtTA3 expression was confirmed by real-time PCR.

Once these MEC lines were validated to stably express the rtTA3 protein, we next transduced these cells with a retrovirus that encodes the required DNA cassette for doxycycline induced ACREB expression (ACREB is regulated by the 3rd gen 'tetracycline response element' promoter, "TRE3G"). This retrovirus also encoded for a fluorescent reporter that enabled us to visualize positively transduced cells by viewing them under a microscope using the appropriate fluorescent filter. Next, in order to obtain a 'pure/homogenous' population, getting rid of any negative cells, we performed fluorescence activated cell sorting. ACREB expression, under doxycycline treatment, was confirmed by probing its effects on canonical CREB regulated transcriptional targets (Fig 1).



RESULTS

Functional validation of ACREB expression



Effects of ACREB expression on MEC cell proliferation and survival

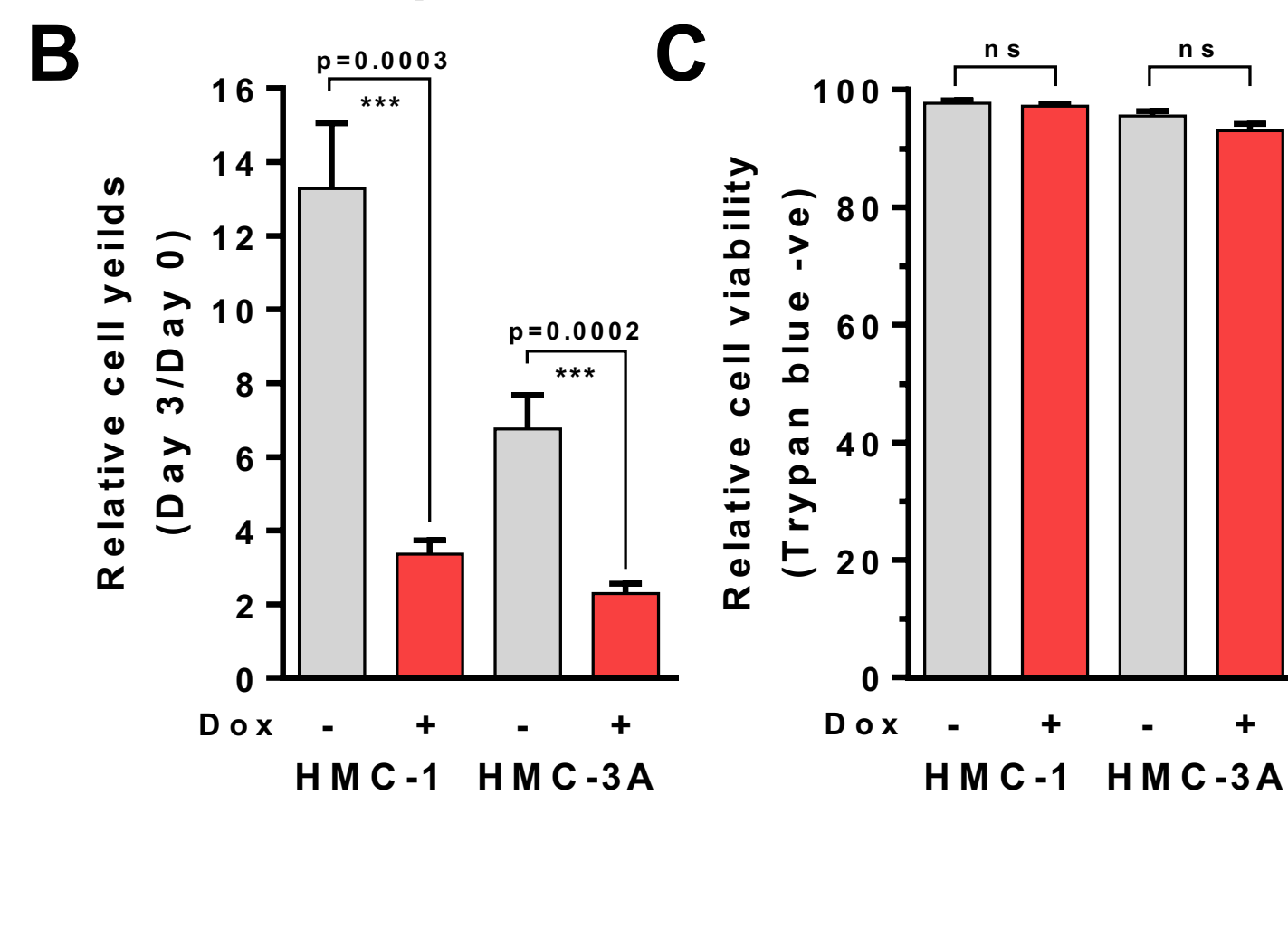
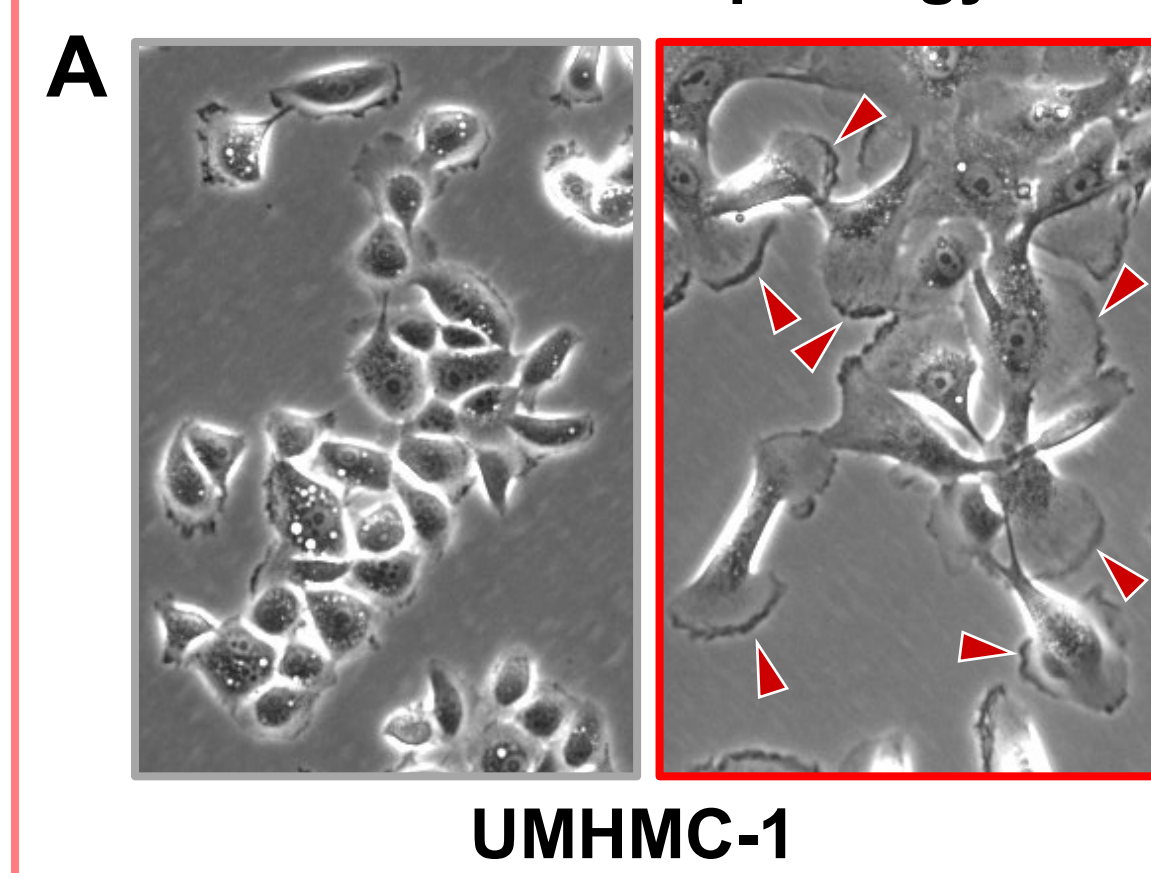


Figure 1: **A-** Blunting CREB activity with ACREB was confirmed by measuring its effects on gene expression levels of NR4A2, a canonical transcriptional target of CREB. As expected, ACREB induction leads to a rapid and robust reduction of NR4A2 expression, as evaluated via real-time PCR assay. **B-** ACREB induction was accompanied with a robust (~2-3 fold) reduction in MEC cell proliferation. **C-** While significant reduction in MEC cell proliferation were seen, no effects on overall cell survival were observed, indicating that cells remain alive and viable.

Effects of ACREB expression on MEC cell morphology



Effects of ACREB expression on MEC cell migration at a single cell level

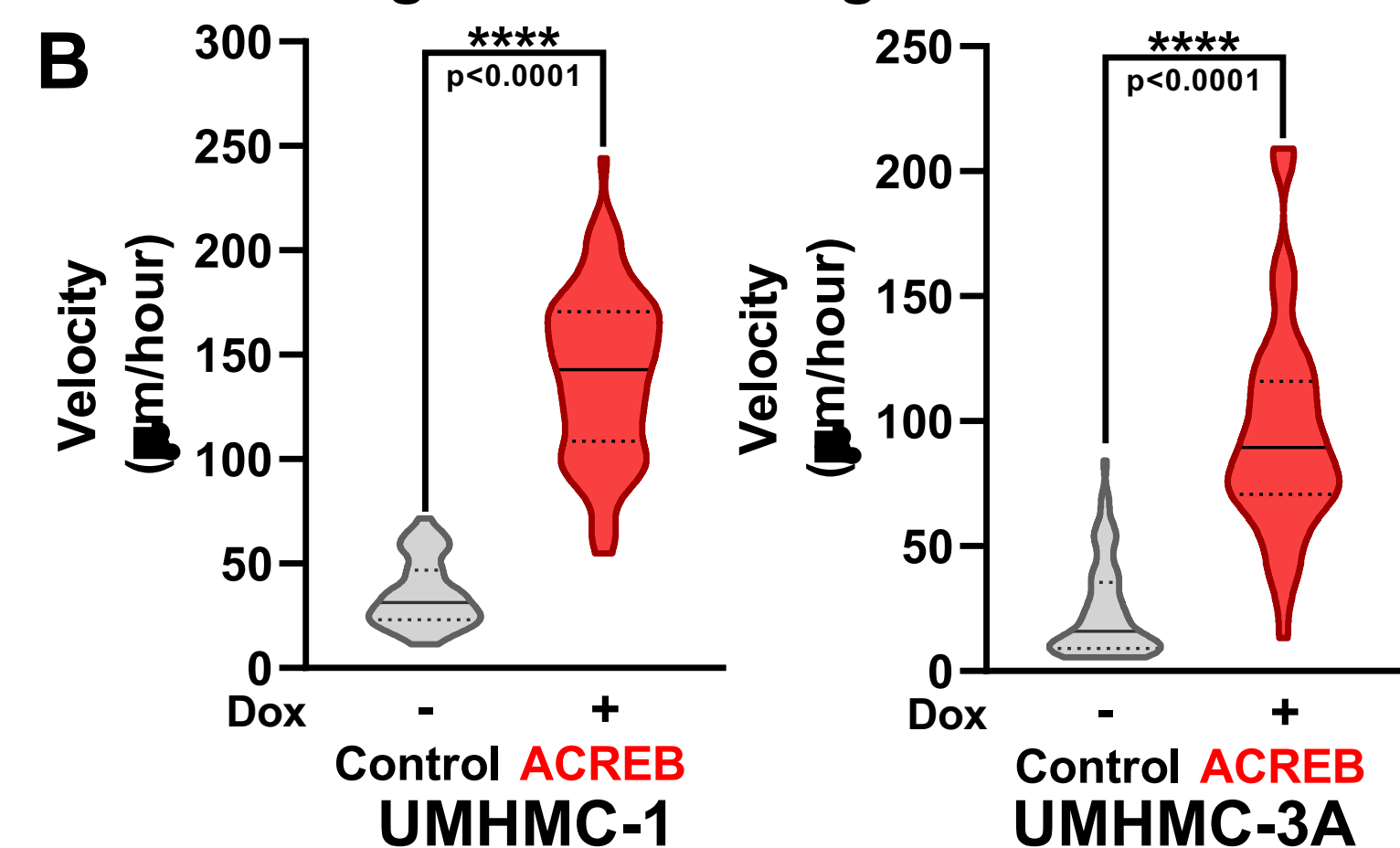


Figure 2: **A-** Prolonged induction of ACREB expression (for 72-120 hours) causes a dramatic shift in MEC cell morphology, wherein the normally epithelial type cells (left) acquired active/ruffled lamellipodia (right-red arrowheads) indicative of a highly motile mesenchymal cell type. **B-** This unexpected epithelial to mesenchymal cell transition (EMT) of MEC cells upon ACREB expression was accompanied with a massive increase in MEC cell mobility, quantified by measuring random single cell migration.

Quantifying the effects of CREB disruption on MEC cell collective migration

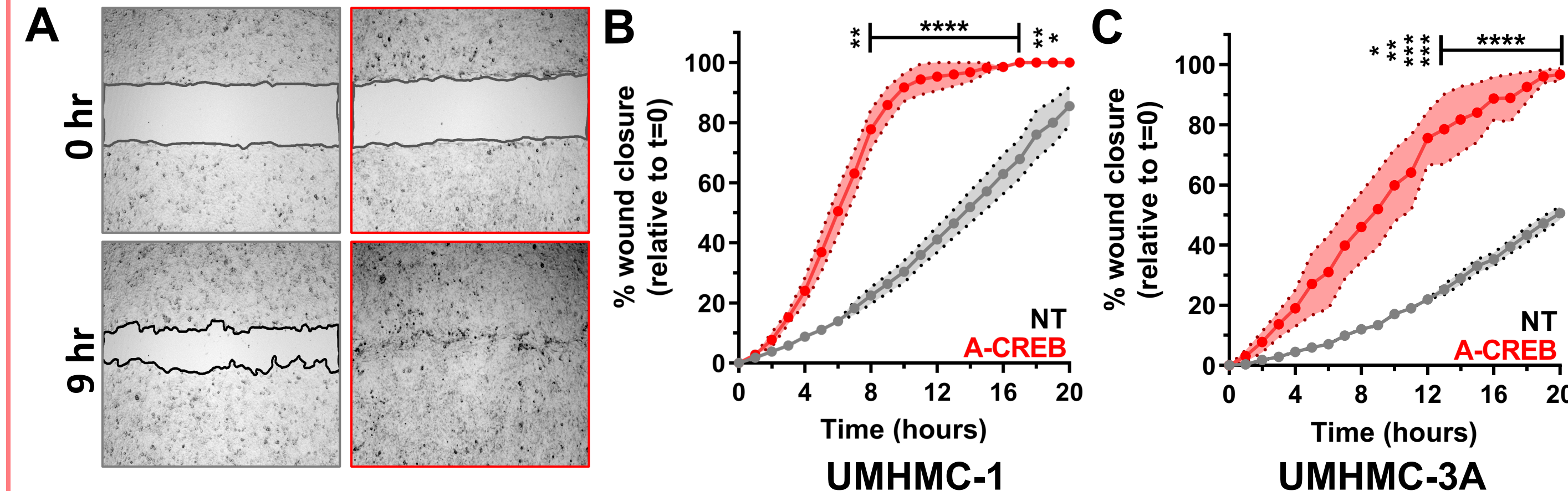


Figure 3: In order to investigate the effects of ACREB induced EMT on MEC cell directional migration, we performed a 'wound' healing assay **A-** MEC cells that have underwent the ACREB induced EMT rapidly migrate towards each other, closing the 'wound' in less than 9 hours compared to normal MEC cells which take >20 hours to fully heal the 'wound'. **B-** Full time course quantification. Thus, ACREB induced EMT state switching of MEC cells and the accompanied increased cell mobility imparts these cells with enhanced wound healing capability, a phenotype typically associated with 'high' grade, aggressive cancer cells.

RESULTS

Quantifying the effects of CREB disruption on MEC cell invasion

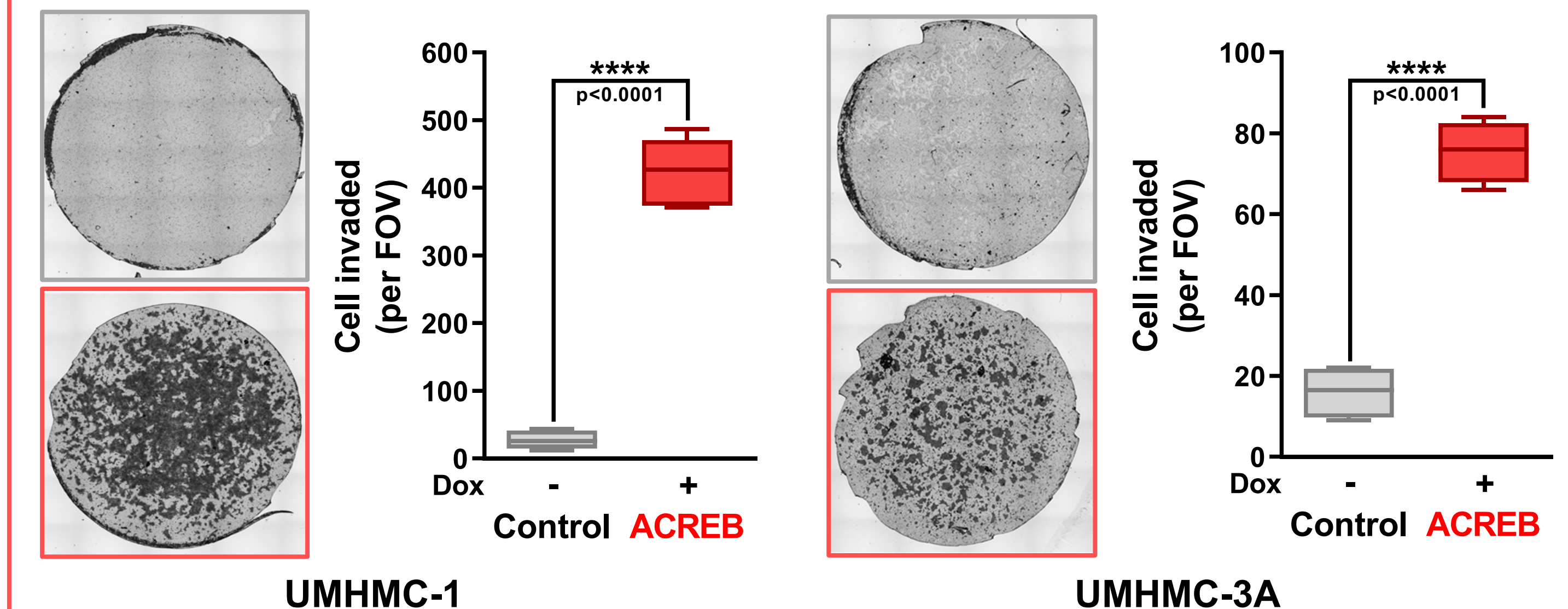


Figure 4: During tumor metastasis, cancer cells first need to migrate out of the primary tumor, enter the circulatory system (could be either blood or lymphatic vessels) and then establish a new tumor at a distal site. A crucial part of cancer cell dissemination, migration out of the primary site, hinges on the cancer cell's ability to break down the surrounding 'extra-cellular matrices' (ECM) in which most tumors are enveloped. Thus, in order to quantify the effects of ACREB induced MEC cell EMT on their metastatic potential we used established ECM-transwell migration assays. We found that CREB disruption in MEC cells imparts these cells with dramatically enhanced invasive capabilities, a key hallmark of highly metastatic, invasive and aggressive 'high' grade tumors.

CONCLUSIONS

1. CREB disruption in MEC cells causes a significant reduction in cell proliferation.
2. However CREB disruption has no effect on MEC cell survival.
3. CREB disruption causes MEC cells to undergo an epithelial-to-mesenchymal transition (EMT), as evidenced by a dramatic changes in cell morphology.
4. This ACREB induced EMT was accompanied with enhanced MEC cell motility (random migration), cell migration (wound healing) and most significantly, with enhanced cell invasion.

Thus, in this study, we have uncovered an unexpected, novel and previously unreported function of CREB towards regulating MEC cell EMT and its role towards in low→high grade transition of MEC tumors.

FUTURE DIRECTIONS

In order to better define the role of CREB towards MEC cell stemness, differentiation and in low→high grade tumor transition, we are going to perform RNA-sequencing on MEC cell in the presence/absence of ACREB. Furthermore, in order to elucidate the functional role of CREB in MEC tumor metastasis, and thus patient survival, we will investigate the effects of CREB disruption towards MEC tumor progression in vivo using murine animal models.

ACKNOWLEDGEMENTS

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