Translational
Radio-
Biology

Hypoxia affects ECM composition, structure and morphology through HIF dependent and independent mechanisms, impairing cell migration in bladder cancer

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INTRODUCTION

- Hypoxia is a feature present in most solid tumours, associated poor prognosis and known to promote cancer metastasis. Bladder cancer is a common disease (>500,000 cases annually) in which hypoxia (low oxygen levels) is heavily prevalent. Up to 70% of patients show some level of expression of classic hypoxia markers (n = 64)[1,2].
- Hypoxia induces cancer metastasis through promoting extracellular matrix remodelling (ECM), acquiring a pro-cancerous fibrotic phenotype. The hypoxic ECM plays a key role in cancer metastasis, providing a scaffold that gives both signalling and structural support for cancer cell migration[3].
- Radiotherapy, standard-of-care treatment in bladder cancer, has synergies with the ECM as it affects cell-ECM interactions through focal adhesions, activating FAK and integrin signalling, which is critical for the induction of epithelial mesenchymal transition[4].

Aim: To perform an omics comprehensive study of hypoxia effects in cancer ECM remodelling, its regulatory mechanisms, effects in cell adhesion and migration, and its synergies with irradiation.

METHODS

- **Cell culture:** UMUC3, J82, RT4 and T24 cell lines were cultured for 7 days in 21% (normoxia), or 0.1-0.2% (hypoxia) O_2 in a Don Whitley Hypoxystation
- **Irradiation:** Cells were irradiated (2 – 8 Gy) with X-rays using a Xtrahl irradiation system.
- **Proteomics:** After decellularization, ECM was collected and analysed through mass spectrometry (MS).
- **ChipSeq:** Gene promotor regions bound to HIF1A/HIF1B were immunoprecipitated and sequenced.
- **Migration:** Cells were seeded onto normoxic or hypoxic ECM, scratched, and migration capacity measured through live microscopy up to 48h.
- **Attachment:** Cells were allowed to attach onto normoxic or hypoxic ECM up to 2h, non-adherent cells removed and % of attached cells estimated by absorbance.
- **Immunofluorescence:** Cells were fixated and antibody-stained for collagen (COL) 1/5 and fibronectin (FN). Nuclear and cytoplasmatic staining was performed with DAPI and phalloidin, respectively.

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RESULTS

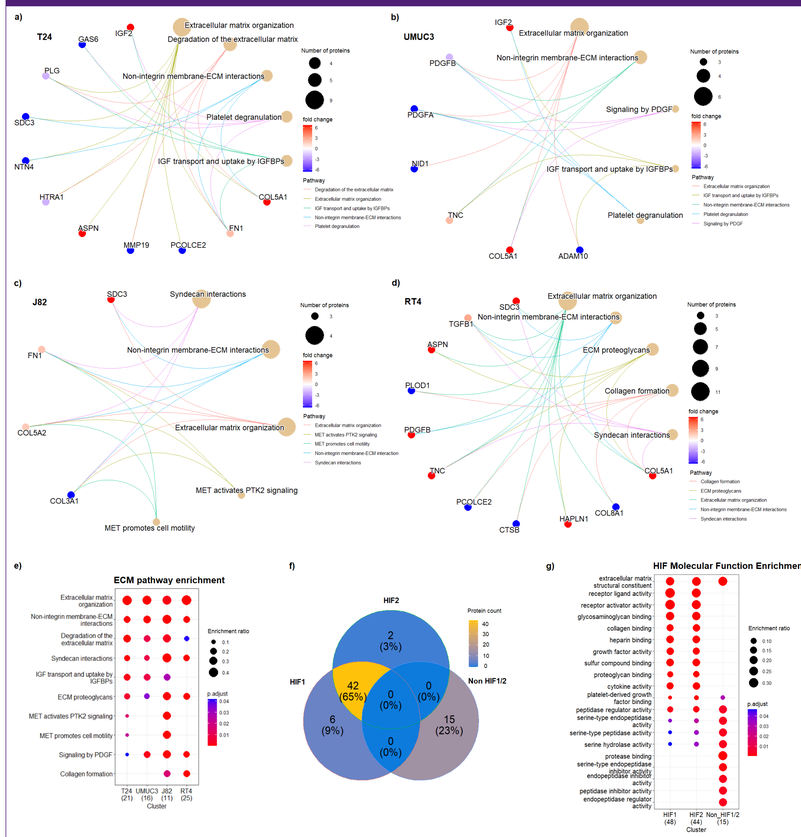

Figure 1: Hypoxia affects the ECM composition in bladder cancer, influencing ECM remodelling and organisation through HIF1/2 dependent and independent mechanisms. The figure shows individual (a-d) and comparative (e) top-5 GO Term Biological Processes (BP) enrichment analyses of 66 significant proteins (fold change ≥2 or ≤-2 and p. adj.<0.05) identified after proteomic analysis of hypoxic ECM *in vitro* samples. The analyses show a common effect of hypoxia in ECM organisation and remodelling across all cell lines. ChipSeq highlighted 65% of those proteins are regulated by both HIF1 & 2, while 15% are regulated by alternative mechanisms (f). Enrichment analysis showed that HIF1/2-regulated proteins are mostly associated with cell/ECM interactions, while protease activity is regulated by HIF independent mechanisms (g).

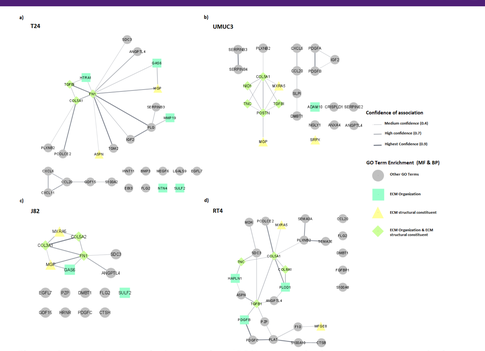

Figure 2: Protein-Protein Interaction (PPI) network shows strong associations among significant proteins associated with ECM Organization and ECM structure. The figure represents those ECM proteins with significant changes (p≤0.05, fold change≥2) in hypoxia for T24 (a), UMUC3 (b), RT4 (c) and J82 (d) cell lines. The PPI network highlights a predominant role of FN and COLs, as well as their association with other "ECM Organization" and "ECM structural constituent" proteins in all 4 cell lines. Confidence score were determined according to the STRING database.

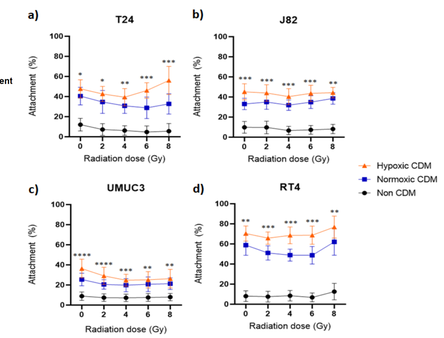

Figure 3: Hypoxia promotes cell adhesion independent of radiation stress. Attachment assays for T24 (a), J82 (b), UMUC3 (c) and RT4 (d) cell lines show hypoxic ECM coating promotes cell attachment increase in all cell lines independently of radiation stress. Each point in the graph represents the mean and standard error of three biological repeats with 20 technical repeats per biological repeat, with * for p<0.05, ** for p<0.01 and *** for p<0.001.

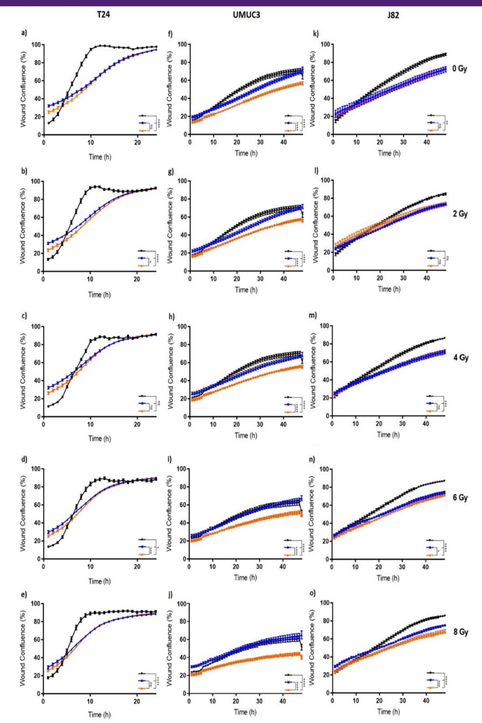

Figure 4: Hypoxic CDM impair cell migration independently of radiation stress (UMUC3) and in a radiation stress dependant manner (T24, J82). Scratch assay for a) T24, b) J82 and c) UMUC3 cell lines comparing their migration on hypoxic and normoxic CDM coating, and a non-CDM coating control for non-irradiated and irradiated (0-8Gy) cells. The graphs (f-j) show impaired cell migration for UMUC3 for the cells seeded onto normoxic hypoxic CDM (0-8Gy) (p<0.0001). Migration impairment was only significant under radiation stress in T24 (2Gy, p<0.05; 6Gy, p<0.001) and J82 (6Gy, p<0.05; 8Gy, p<0.001). Cells on non-CDM control plates had a higher rate of migration across all cell lines, except for irradiated UMUC3 (6-8Gy). Significance was assessed using a 2-way ANOVA, with * for p<0.05, ** for p<0.01, *** for p<0.001 and **** for p<0.0001). Each datapoint represents the average + SEM of 3 independent experiments including 12 technical replicates per experiment.

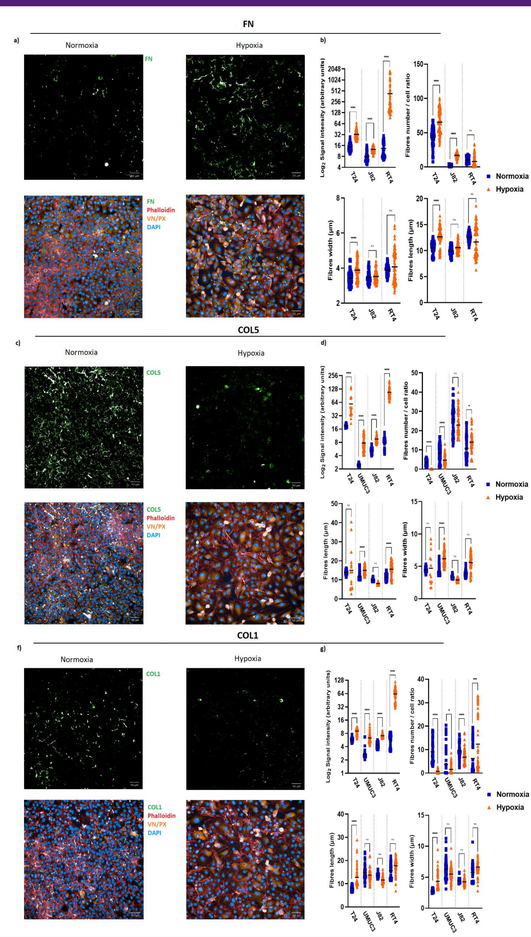

Figure 5: Hypoxia affects the morphology and number of ECM fibres. The figure shows Immunofluorescence images of T24 cell line staining for FN (a), COL5 (c) or COL1 (e) (green channel) under hypoxia stress. Staining was also performed for VN/PX (yellow channel), actin (red channel) and nucleus (DAPI). Bar plots represent the fibres intensity, number, width, and length for FN (b), COL5 (d) and COL1 (f). Analysis confirms a significant increase in signal intensity for FN, COL1 and COL5 under hypoxic stress. Significant increase of fibres numbers was seen for FN. However, COL1 and COL5 show a significant decrease in fibres number. Finally, a significant increase in fibres length is also seen for FN, while COL1 fibres had a significant increase in both fibres length and width. Significance is defined as p. value < 0.05 and at least 20% fold-change respect normoxia control, with * for p<0.05, ** for p<0.01, *** for p<0.001 and **** for p< 0.0001. For each experiment, n = 3 biological repeats, with n = 20 technical repeats for each biological repeat.

CONCLUSIONS & FUTURE WORK

- Hypoxia induces compositional changes in the bladder cancer ECM in vitro mainly affecting structural ECM proteins.
- Hypoxia promotes morphology changes (increased length and width) and also affect fibres number (increase FN, decrease COL) for FN and COL fibres.
- Hypoxic ECM increases cell attachment (independently of radiation stress) and impairs cell migration (which can be dose dependant of radiation stress)
- Future studies should focus in the role of focal adhesion molecules and their interaction with an hypoxic ECM

Proposed model:

Long term hypoxia (0.2% O_2) destroys ECM COL fibres but induces FN fibres

- FN & COL available (T24, J82) → Compensatory effect; No differences in cell migration unless irradiation is involved
- Only COL available (UMUC3) → Decreased migration in hypoxia; no fibres available for cells to migrate

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