Inflammation in the Tumor Microenvironment: Experimental Models of Metastatic Melanoma Therapy

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Melanomas are heterogeneous tumors comprised of distinct cell subtypes that collectively form a single 'organ.' Failure to effectively target genetic diversity within this malignant organ underlies stalled tumor regression. This is referred to as resistance, wherein the tumor organ or microenvironment (TME) no long shrinks in response to therapy. Instead, invasive melanomas outgrow and secrete signals to alter the TME thereby promoting progression and dissemination. This inflammatory process shares many features in common with wound healing, and chronic inflammation has become an emergent hallmark of cancer. This dissertation establishes that transforming growth factor-β (TGFβ) release is an essential mediator of the persistent inflammation in the TME. In response to therapeutic stress, melanoma is shown to upregulate expression of pro-inflammatory cytokines and TGFβ. These molecules concomitantly antagonize the stroma leading to the activation of gene networks associated with wound healing and epithelial-to-mesenchymal transition (EMT). Here, TGFβ ligands are shown to transmit signals to fibroblasts to promote transformation into contractile myofibroblasts, referred to as cancer associated fibroblasts (CAF). These α-smooth muscle actin (αSMA^{+}) CAFs transiently accumulate in wounds and sites of chronic inflammation. Within these inflamed sites CAFs secrete and synthesize 'wound-associated' extracellular matrix (ECM) which blunt the anti-tumor response to therapies. Dysregulated ECM promotes drug resistance via growth factor binding (*e.g.* TGFβ) and cell-ECM signaling that promotes invasion of dense tissue melanoma spheroids. Here, matricellular tumor-ECM is shown to contain the matrikine tenascin-C (TNC). Tenascin-C has been experimentally

shown to bind epidermal growth factor receptor (EGFR), promote malignant invasion, and promote motility and survival. Cocultures therapy inflamed melanomas are shown to stoke (αSMA^{+}) CAFs. Clinically relevant inhibitors of the TGFβ receptor were used to perturb TGFβ signaling between the tumor-and-stroma. This led to the suppression of CAF transformation and limited *in vitro* outgrowth of melanoma via drug sensitization to mitogen-activated protein kinase (MEK) inhibitors. *In vivo*, a combination strategy to inhibit TGFβ activation of mouse CAFs results in MEK suppression of melanoma outgrowth and limits metastasis. Thus, combined targeting of the tumor-and-stroma limits cytotoxic cell stress and CAF remodeling of the inflamed TME and prevents deposition of progression associated ECM molecules.

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Preface

To my family and friends:

Your love and support have rivaled the biggest challenges, thank you.

To my committee:

Your guidance and mentorship have made me a better person and scientist.

To Alan:

You are mentor and friend in the absolute sense.

A true Renaissance man.

Thank you.

1.0 Introduction

The diagnosis of melanoma continues to increase in frequency and is readily curable in early-stage cutaneous disease. Roughly nine in 10 of the more than one million individuals with melanoma will survive. However, survival is drastically reduced following dissemination (5-year survival rate of 22%, **SEER**). While there has been significant progress in the detection and treatment of melanoma, the most effective cure remains surgical resection. Inoperable metastatic disease accounts for nearly all melanoma-related deaths and is virtually incurable. Advances in the understanding of melanoma plasticity and the progression from healthy to pathological tissues that form the tumor microenvironment (TME) have offered new insights toward developing more effective therapeutic strategies.

1.1 The Metastatic Cascade

Persistent exposure to environmental and genetic insult drives melanocyte transformation into malignant melanoma. These localized and even regional sites of disease are curable if detected and removed. However, progression leading to dissemination often results in the formation of intractable metastases with dismal prognoses. In metastatic disease, disseminated melanomas escape the primary tumor and immune surveillance, transit the vasculature, and colonize ectopic tissues. This highly inefficient process will persist if a small fraction of escaped cells successfully enters into spontaneous quiescence or dormancy in the ectopic tissue (**Fidler JI et al., 2003; Talmadge JE et al. 2010; Hanahan D & Weinberg RA 2000, 2011**). These minimal residual

melanomas remain dormant, however transient, prior to an inflammatory stimulus that promotes the emergence of metastatic disease (**Figure 1**).

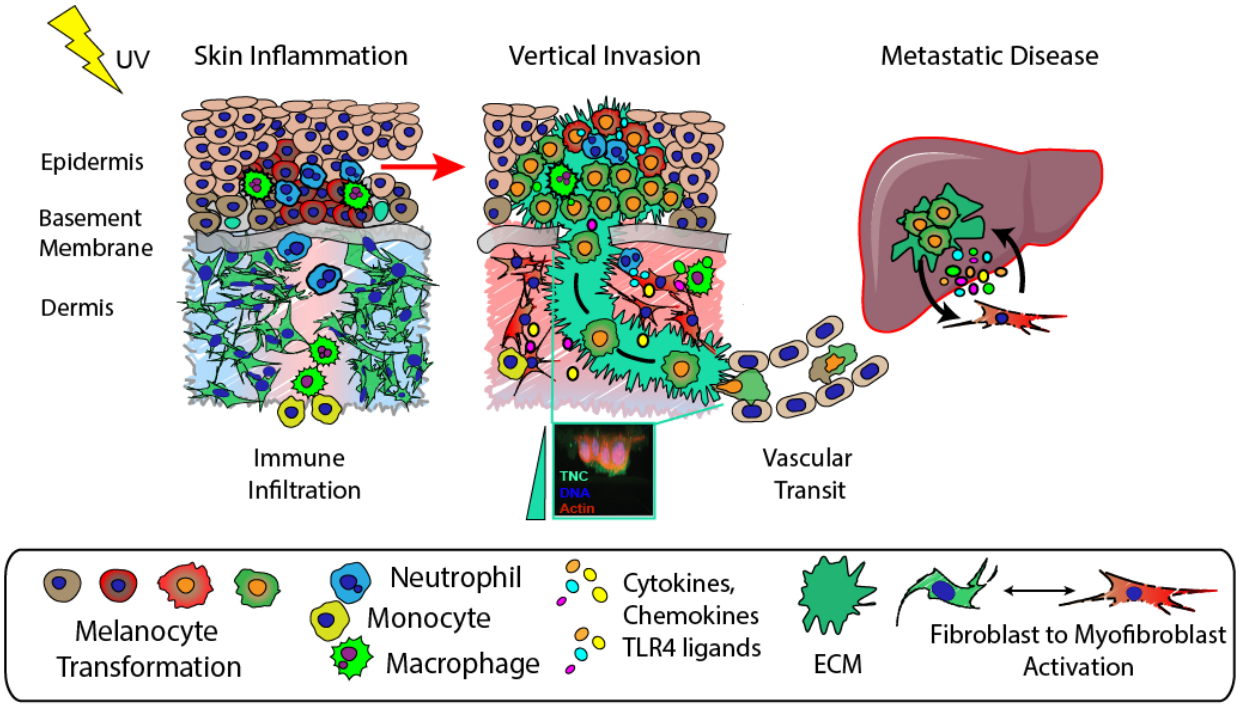

Figure 1: Schematic of melanoma dissemination and colonization

Skin Inflammation: Chronic environmental damage (UV) coupled with DNA damage and mutations drive cellular death and the release of damage associated proteins (DAMPs). These bioactive signals recruit myeloid derived cells (immune infiltration) to clear apoptotic cells. Immune intravasation drives inflammation mediated by cytokines, chemokines, and proteolytic cleavage of extracellular matrix. **Vertical Invasion**: Stromal fibroblasts are inflamed as melanoma invades along a tenascin-C (TNC) front, during dissolution of the skin basement membrane. Therapeutic treatment to halt progression drives reciprocal signaling and TLR4 driven immunosuppression. A pro-tumorigenic therapy induced secretome promotes ECM deposition and a matrisome that promotes angiotropism and escape from the primary tumor. Circulating melanoma cells transit the vasculature to ectopic organs. **Metastatic Disease**: Small fractions of tumor cells colonize ectopic tissues and undergo dormancy where they remain immunosilent prior to reemergence.

Metastasis is still the predominant cause for most cancer-related deaths – despite clinically confirmed remission or cure (**Chaffler et al., 2011**). The establishment of metastatic melanoma is ominous as frontline treatments such as checkpoint inhibitors quickly lose efficacy in comparison to responsive primary lesions (**Balch CM et al. 2009**). A major obstacle for the eradication of melanoma is tied to the genetic basis of individual clones, which can be grouped into multiple genetic subtypes (BRAF, RAS, NF1 and Triple Wild type). Mutant BRAF tumors typically contain amplifications of melanocyte inducing transcription factor (MITF) and $BRAF^{V600E}$, which is present in roughly half of melanomas. Mutant RAS tumors are typified by activation of mitogen-activated kinases (MAPK) and serine/threonine kinase (AKT) pathways, which is often activated in response to therapeutic targeting. Mutant NF1 tumors occur in aged patients and often show high mutational burden, while triple wild-type tumors exhibit copy number variations (CNV) indicative of mutational events but paradoxically lack an ultra-violet (UV) signature (**Cancer Genome Atlas Network, 2015**). Treating these tumors is therefore complex. Single-cell RNA sequencing revealed that a majority of melanomas contain multiple subtypes within single lesions **(Tirosh I et al. 2016**). Thus, FDA approved inhibitors vemurafenib, dabrafenib, and trametinib which target these genetic drivers often display a strong initial response that is seldom durable in terms of the overall response rate (ORR) with relapse within ~5-7 months and eventual death **(Gadiot J et al., 2013; Haferkamp S et al. 2013; Hauschild A et al., 2012; Lee JT et al., 2010; McArthur GA et al., 2013).** Reduced therapeutic penetrance is common in metastatic melanoma despite effective regression of the primary tumor. This paradigm is likely linked to the incomplete understanding of how metastatic tumors coevolve within distinct microenvironments to acquire resistance (**Wagle N et al., 2011; Van Allen EM et al., 2014**).

1.2 The tumor microenvironment promotes progression

Melanoma is non-cell autonomous and requires hospitable microenvironments for progression and survival. Our foundational model views tumors as an organ wherein cancer survival is driven by modifications in cell and extracellular matrix (ECM) signaling. The TME is comprised of non-malignant stromal and immune cells, ECM, and signaling molecules. This cellular ecosystem generates heterotypic interactions to support cancer proliferation, invasion, and drug resistance (**Junttila MR et al. 2013; Qain BZ et al., 2010; Joyce J et al., 2009; Kalluri R et al., 2006; Egebald M et al., 2010**). In this regard the TME is vital for progression to lead to metastasis. Reciprocal stromal and cancer signaling can activate cancer intrinsic epithelial-to-mesenchymal transition (EMT) and removal of stromal cells from the TME results in a loss of immunosuppressive gene expression (**Yang et al., 2008; Tirosh I et al., 2016**). This suggests that bidirectional signaling in the TME is essential for the stepwise progression of melanomas from low-to-high grade malignancies.

1.2.1 The complicated biological origins of CAFs

Of the several possible cell-cell interactions in the TME, few precede melanomas and cancer associated fibroblasts (CAFs). CAF signaling manifests via cell-cell contacts, adhesion molecules, and signaling factors such as growth factors, cytokines chemokines, ECM, MMPs, protease inhibitors, and lipids (**Ruiter D et al., 2002; Píerard GE et al., 2012; Werner W et al., 1997**). The precise origin of CAFs is unknown though the most likely source is suspected to derive from tissue resident fibroblasts. However, transdifferentiation of pericytes or bone marrow derived mesenchymal cells (MSCs) have not been excluded despite the necessity to first undergo

endothelial /- epithelial-to-mesenchymal (Endo-/ EMT) (**Gascard P et al., 2016; Kullari R, 2016; Xiao et al., 2015**). CAFs are morphologically distinct in comparison the spindle-like normal counterpart. CAFs appear larger with more focal adhesions and exhibit enhanced proliferative and migratory capacity to epigenetic and protein expression (**De Wever, O et al., 2008; Saadi, A. et al., 2010; Ma, XJ et al., 2009**). Thus, the inherent lack of CAF ontogeny lends to the complexity of these heterogeneic stromal populations. Consequently, a plethora of signaling pathways have been implicated in CAF conversion and activation.

1.2.2 Signals that awaken the tumor stroma

The TME is replete with growth factors and paracrine signals which can functionally activate fibroblast conversion to CAFs. Of the many potential candidates, transforming growth factor-β (TGFβ) has been implicated as a central player in the development of CAFs. The TGFβ superfamily consists of TGFβ1, 2, and 3 which bind to the cognate serine/threonine protein kinase TGFβ receptor (TGFβR type I/II) to augment the phosphorylation of intracellular SMAD transcription factors. Activated SMADs then transit the cytosol to form SMAD complexes which ultimately localize to the cell nucleus and bind hundreds of genomic loci to drive transcriptional activation or repression (**Figure 2**) (**David CJ et al., 2018**).

Transforming growth factor-β has multiple contextual roles in wound healing and fibrosis which are relevant to CAF activation, though whether TGFβ is tumor suppressive or promoting remains controversial. Co-implantation of breast-tumor xenografts with resident tissue-matched fibroblasts resulted in healthy fibroblast conversion to CAFs. This transformation was driven by

autocrine TGFβ and stromal cell-derived factor 1 (SDF-1) signaling loop (**Kojima Y et**

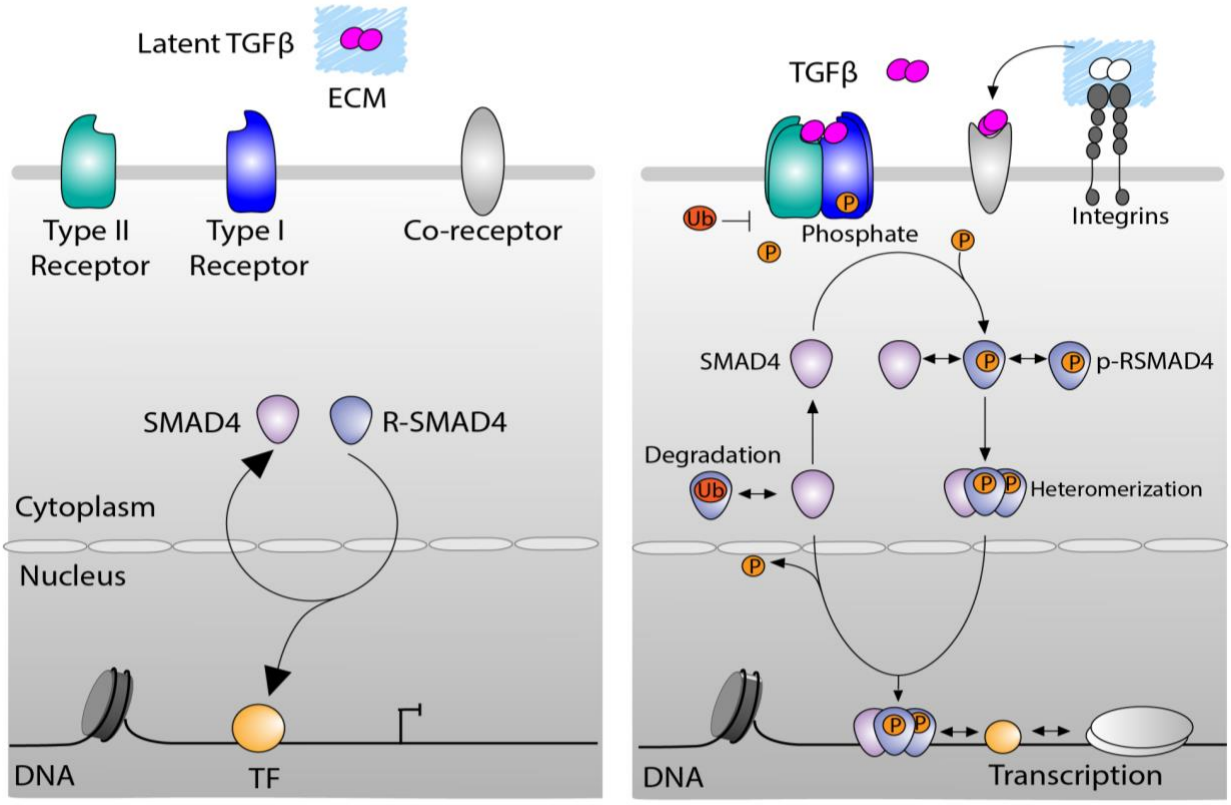

Figure 2: The TGFβ signaling pathway

In the basal state **(LEFT)**, TGFβ is sequestered in the extracellular matrix (ECM) in a latent inactive state, while the serine/threonine transmembrane type I/II TGFβ receptors exist as inactive monomers (allosteric inhibition). Mothers against decapentaplegic homologue (SMAD) proteins in the cytoplasm (SMAD 1, 2, 3, 5, 8) cycle as inactive monomers while SMAD4 transits in-and-out of the nucleus. **(RIGHT)** Upon activation, ligands are released in the tissue microenvironment (allosterically by integrins) and bind TGFβ receptors to form ligand-receptor complexes with type II receptor activation type I subunits by phosphorylation. Activated type I/II receptor complexes phosphorylate SMADs which phosphorylate and from heterotrimer complexes with SMAD4 (R-SMADs). Nuclear localized SMAD complexes bind to hundreds of genomic loci activating transcription factors to initiate transcription. Inhibitory cofactors drive degradation of R-SMADs to end transcription in the nucleus while inhibitory SMADs block phosphate from binding the receptor complexes in the cytoplasm. Ubiquitin (Ub) is recruited to shuttle SMADs for proteasomal degradation. Receptor dissociate and return to basal state in the absence of ligand activation. Modified figure reproduced from (David CJ et al., 2018).

al., 2010). CAFs have also been shown to reciprocally drive EMT in breast cancer via TGFβ-SMAD signaling, while SDF-1 which maintains the CAF phenotype, is reported to enhance breast

cancer invasion and migration (**Yu, Y et al., 2014; Al- Ansari, MM et al., 2013**). However, the release of TGFβ is not exclusive to fibroblasts. This begs the question of sources of TGFβ in the TME and how expression may differ among subtypes of melanoma populations. Normal fibroblasts, presumably lacking activation of TGFβ-SDF1, are reported to suppress tumor growth (**Chen X et al., 2018**). This well-established paradigm is bi-directional. Co-engraftment studies with CAFs expressing TGFβ is reported to drive human mammary epithelial cell hyperplasia whereas inoculation with normal fibroblasts resulted in tumor stasis (**Kuperwasser C et al., 2004; Picard O et al., 1986**). Despite these foundational studies, there is a lack of clarity surrounding the mechanisms of whether TGFβ is pro-tumorigenic, which is exemplified by a study reporting that TGFβRII suppression in CAFs led to tumor expansion (**Busch, S. et al., 2015**). Therefore, TGFβ signaling in CAFs and how this impacts melanoma progression is still open ended.

1.2.3 CAF are foundational in cultivating the TME

The presence of pro-inflammatory cytokines, growth factors and exosomes within the TME converge to drive fibroblast transformation into hypercontractile myofibroblasts. Upon transformation, CAFs release a bioactive secretome and exhibit a greater synthetic capacity, which combine to promote progression and reshape therapeutic responses (**Sahai E, et al., 2020; Bhome, R. et al. 2017**).

The epigenetic and microenvironmental activation of CAFs drives the release of multiple matrix metalloproteases 1, 2, 3, 9, 12, 19 (MMPs), protease inhibitors (TIMPs), in addition to a host of tumor supportive chemo-and-cytokines (e.g., *abbrev*., TGFβ, EGF, FGF, VEFA, PDGF, HGF and IL6) which have pivotal roles in cultivating the TME (**Kullari R, 2016**). The potency of these molecules is typified by hepatocyte response to non-malignant release of IL6, which was

shown to drive a pro-metastatic niche in the liver; the most common metastatic site (**Lee JW et al., 2019**). This is particularly salient given that physiologic stress, such as an invading tumor, has the potential to trigger fibroblast release of IL-6 and Activin A (TGFβ family) (**Fordyce, CA et al., 2010, 2014**).To expand, production of vascular endothelial growth factor (VEGF) endows CAFs with pro-angiogenic capacity (**O'Connell, JT et al., 2011**), MMPs to promote ECM remodeling, epidermal growth factor to promote tumor invasion and cell motility (**Grahovac et al., 2014),** platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) to stimulate CAF proliferation, and the synergistic signaling of hepatocyte growth factor (HGF) combined with TGFβ, IL6 to promote drug resistance to $BRAF^{V600E}$ and EGFR inhibition (**Kumari N et al., 2016; Wilson TR et al., 2012; Wang W et al, 2009; Straussman R et al., 2012**). Interestingly, the CAF secretory phenotype is largely maintained *in vitro* in the absence of the tumor and TME (**Orima et al., 2005**). However, refined genetic analysis of CAFs outside of the TME have revealed that pro-tumorigenic gene networks are lost (**Tirosh et al., 2016**). This implies the importance of establishing a tumor niche to sustain CAFs which in turn maintain the TME. This notion is underscored *in vivo* with mice lacking S100A4 which inhibits myofibroblast differentiation and prevents metastasis (**Grum-Schwensen, B. et al., 2005**). Thus, the disruption of bioactive CAF signaling should hypothetically stall progression via elimination of the TME.

1.2.4 CAFs modify ECM to reinforce tumor progression

Cancer associated fibroblasts are the presumptive key ECM producing and remodeling cell population within the TME. The ECM is an ornate network of cross-linked proteins that relays biophysical and biochemical signals to contextually regulate tissue development, cell proliferation, survival, and motility (**Naba A et al., 2012**). Despite transient dysregulation during wound healing,

the persistence of aberrant ECM contributes to fibrotic disease and malignant progression. In this context the ECM is three-dimensional bioactive scaffold that acts as a foundation for cells, providing mechanical stability and physiochemical signals via sequestration of growth factors and cytokines (**Pickup M et al., 2014**). However, the ECM present in solid tumors differs dramatically from healthy tissue.

Tumor ECM is rich in fibrillar collagen (COL-1) but also type III, IV and V, hyaluronan, fibronectins, laminins which comprise basement membranes, and wound response matricellular protein tenascin-C (TNC) (**Ronnov-Jessen, L et al., 1996; Rodemann, HP et al., 1996; Tomasek, JJ et al., 2002**). This complex ECM network is regularly pruned and remodeled primarily due to CAF proteases driving the release of cryptic growth factors such as VEGFA to promote angiogenesis. However, the disorganization of the ECM impacts the leakiness of vascular beds leading to hypoxia, a feature that is associated with adverse outcomes (**Acerbi I et al, 2015; Gilkes D et al., 2014; Levental KR et al, 2009; Lu P et al, 2012; Naba A et al, 2014**). Other important molecules such as TGFβ and macrophage chemotactic protein 2 (CCL2) are released during ECM remodeling. These molecules impact melanoma invasiveness through TGFβ driven EMT and recruit immune cells which are reprogrammed due to immunosuppressive CAF activity (**Flavell, RA et al., 2010; Kim JH et al., 2012; Yang, X. et al., 2016**). Thus, excessive ECM is pathological, yet there is only a facile understanding of how key proteins impact progression

1.3 Tumor ECM is dynamically modified in melanoma

As melanoma tumors expand the ECM is the first barrier to spreading. Vertically invading melanomas must (1) polarize the cell body, (2) utilize integrins to develop focal adhesions to the

ECM (3) secrete proteases and matricellular proteins at the leading edge to disrupt the basement membrane, and lastly (4) retract the lagging edge to transit into the dermis. There melanomas interact with tissue-resident fibroblasts which are converted to CAFs. In addition to ECM synthesis, CAFs also produce ECM modifiers (e.g. lysl oxidase, LOXL2) that crosslink ECM proteins thereby stiffening tumors, and MMPs which proteolytically degrade matrix to permit invasion (**Nguyen EV et al., 2019; Tang X et al., 2016; Mohammadi H et al., 2018**). For example, sromelysin-1 (MMP3) is released by CAFs to actively cleave cell-cell adhesion molecule epithelial cadherin (E-cadherin) to promote cancer EMT (**Lochter, A. et al., 1997**). These mesenchymal cells are highly motile due to the tumor ECM, which is characterized by decreased cell adhesion, which is a microscale feature that favors cell motility (**DiMilla PA et al, 1993**). Interestingly, TGFβ has been experimentally linked to pro-tumorigenic CAF recruitment to promote tumor ECM that correlates to increased metastasis (**Dumont N et al., 2013; Madsen CD et al., 2015; Calon A et al., 2012; Lohr M et al., 2001; Aoyagi Y et al., 2004**). Here, the focus is on TGFβ and how expression of this molecule is impacted by tumor suppressive ECM molecule decorin (DCN), and tumor promoting matricellular ECM tenascin-C (TNC).

1.3.1 Tumor inhibiting ECM

The ECM in healthy mature tissues presents and signals to cells differently than the disorganized and immature pathological ECM that is often characteristic of malignancy. As the ECM is bioactive, this substrate is capable of regulating multiple aspects that govern the hallmarks of cancer which include proliferation, circumvention of growth suppression, apoptotic resistance, initiation of invasion, dysregulation of cellular energetics, immune escape, and chronic inflammation (**Pickup M et al., 2014, Hanahan D et al., 2011**). The term matricellular or

matrikine has been proposed to define ECM proteins that contain domains which can signal within the tissue microenvironment (**Bornstein P et al., 2002**). These complex proteins are often large, spanning multiple to hundreds of kilodaltons. Within these large spans exist cryptic sites. These sites are enzymatically exposed by MMPs secreted by invasive melanomas. Additionally, the intravasation of malignant cells produces mechanical forces via integrin contractions of the ECM that drive the release of adsorbed growth factors (**Maquart FX et al., 1999; Davis GE et al., 2000**). However, these sites can be tumor suppressive or promoting within the context of the tissue microenvironment.

Mature ECM is predominantly comprised of organized collagens which bind and anchor molecules that suppress proliferation, migration and promote differentiation (**Grahovac J et al., 2014**). These proteins often appear in the end phases of tissue development or in the resolution phase of wound healing (**Wolf K et al., 2009**). Failure to clear immature matrix results in tissues with similar dynamics to the TME, with both conditions proceeding as incomplete chronic healing. Thus, it is essential to understand how the ECM in tumors coevolves within the context of the reciprocal signals of the tumor and the stroma. Here, the focus is on decorin, a well characterized ECM molecule that has been tested as a therapeutic modality against multiple cancers and is shown here to inhibit melanoma invasion (**Neill T et al., 2016**).

1.3.1.1 Decorin

Decorin is a small leucine rich proteoglycan (SLRP) that is synthesized by stromal fibroblasts and smooth muscle cells. This small SLRP decorates collagen leading to readily accessible inhibitory interactions with growth factor receptors such as EGFR, Met, and PDGF (**Zhang G et al., 2006; Iozzo RV et al., 1999; Goldoni S et al., 2009; Merline R et al., 2011**). Subsequently the protein core and single chondroitin/dermatan sulfate glycosaminoglycan chain

attached to a serine near the N terminus, facilitates multiple binding interactions that endow DCN with anti-tumor capacities (**Keene DR et al., 2000**). There are two sites within the DCN core that bind and sequester TGFβ. Decorin has also been described as the "guardian from the matrix" and is a pan-kinase inhibitor which blunts tumor cell migration via the inhibition of growth factor receptors. Interestingly, DCN also leads to E-cadherin expression in opposition of tumor EMT (**Neill et al., 2012; Hildebrand A et al., 1994; Bi X, et al., 2012; Sofeu Feugaing DD et al., 2013**). Moreover, quiescent fibroblasts produce DCN while actively proliferating fibroblasts have reduced DCN expression which is more in line with the production of pro-tumorigenic matrix. In tumors DCN is found in the peritumor stroma but is excluded within the invading tumor where immature matrix such as TNC is high and cells are positive for EMT (**Franz M et al., 2006; Grahovac et al., 2013; Hlubek et al., 2007**). Thus, redistributing DCN into the invading tumor has therapeutic potential to inhibit invasion by homeostasis of the ECM.

1.3.2 Tumor promoting ECM molecules

The ECM is an amendable substrate that is modified in response to the cells that dwell on the surface. Normal tissues tightly regulate the release of growth promoting factors that activate proliferation via induction of the cell-cycle to maintain homeostasis. Organize tissue architecture regulates cell cycle progression by promoting cellular attachment to the ECM (**Pickup et al., 2014**). In contrast, malignant cells secrete modified matrix to disrupt adhesion, enabling escape from the proliferative control of the ECM (**Zahir N et al., 2004**). The acquisition of tumor-ECM secretion is activated by oncogenic RAS which signals via ERK to induce anchorage independence that is linked to altered ECM expression (**Pattabiraman PP et al., 2010**). During this process the release of growth factors and inflammatory ECM activate the quiescent stroma. This activation

cascade drives an exuberant expansion of cells and the formation of the TME which appears to stiffen in solid tumors overtime, a biomechanical feed-forward process that drives progression (**Provenzano PP et al., 2008; Wells RG., 2008**).

In response to injury or invasion, stromal cells activate developmental ECM gene expression to initiate repair (reviewed by Sylakowski et al. 2020, Appendix B). These transiently expressed proteins decrease ECM density and adhesiveness to promote cell migration. Malignant cells exploit the intermediate adhesiveness of matricellular proteins, displaying decreased dwelling time and escape from proliferative control (**DeMilla et al., 1991, 1993**). Matricellular proteins, Tenascin-C (TNC), thrombospondin (TSP), laminins, and secreted protein-rich in cysteine (SPARC) are increased in sites of inflammation and in the TME (**Grahovac et al., 2014**). Additionally, TGFβ has paradoxical roles in progression wherein this molecule is tumor suppressive during early progression via anti-proliferative signaling. In late stage progression, TGFβ released from inflammatory immune cells and a reactive stroma synthesizing tumor ECM is reported to activate malignant EMT (**Massagué, 2008; Leight J et al., 2012**). Moreover, TGFβ is hypothesized to recruit and stimulate inflammatory cells to the invasive tumor margins. Here these cells secrete MMPs which facilitate malignant amoeboid invasion (**Talmadge et al., 2010, McGowan et al., 2009, Aguirre-Ghiso, 2007; Townsen et al., 2006 and Fidler, 2003**). Thus, the tumor promoting ECM molecules are crucial to promote invasion. Here, the role of TNC is emphasized as it shown to promote melanoma invasion.

1.3.2.1 Tenascin-C

Tenascin-C is a multi-domain glycoprotein with diverse functions in developmental contexts, wound healing, and malignancy (**Giblin S et al., 2014**). Structurally, TNC is a homodimer of homotrimers linked by disulfide bonds within the N-terminal trans-assembly

domain (TA-Domain), giving rise to a hexabrachion structure (**Jones SF et al., 2000; Hancox RA et al. 2009**). Each TNC monomer is composed of domains including the TA, EGF-Like (EGFL), Fibronectin type III (FNIII) and fibrinogen globe (FBG). The FNIII domains are subject to exonic alternative splicing resulting in products of molecular weights between 180-330 kDa (**Midwood KS et al., 2009**). The FNIII and FBG domains are considered adhesive due to integrin binding (**Hancox RA et al. 2009; Saito Y et al., 2007; Joshi P et al., 1989; Grahovac J et al., 2014; Fowler WE et al., 1979; Leahy D et al., 1992; Gulcher JR et al., 1989**). Conversely the EGF-L domains are anti-adhesive and function as an ultra-low affinity ligand for EGFR, measured at K_D = 74μM (**Swindle CS et al., 2001; Iyer AK et al., 2007**). However, TNC matrix tethering augments the strength of TNC-EGFR binding, driving 'high avidity/low affinity' restriction of EGFR to the cell membrane [enriched in lamellipodia] (**Shao H et al., 2015**). Restriction of EGFR in this manner shifts the balance of cell adhesion/contraction to favor migration in a mesenchymal state or reversion to amoeboid for 3D invasion, in place of proliferation (**Grahovac J. et al. 2012; Zaman MH et al., 2006; Friedl P et al. 1997; Friedl P et al., 2012; Iyer AK et al., 2008**). The low cell adhesion to TNC has been shown to promote invasion by both MMP and protease independent mechanisms (**Tremble P et al., 1994**). In this regard TNC and the adhesion

modulating properties of this matricellular protein have been correlated to pro-metastatic activity (**O'Connell, JT. et al., 2011**).

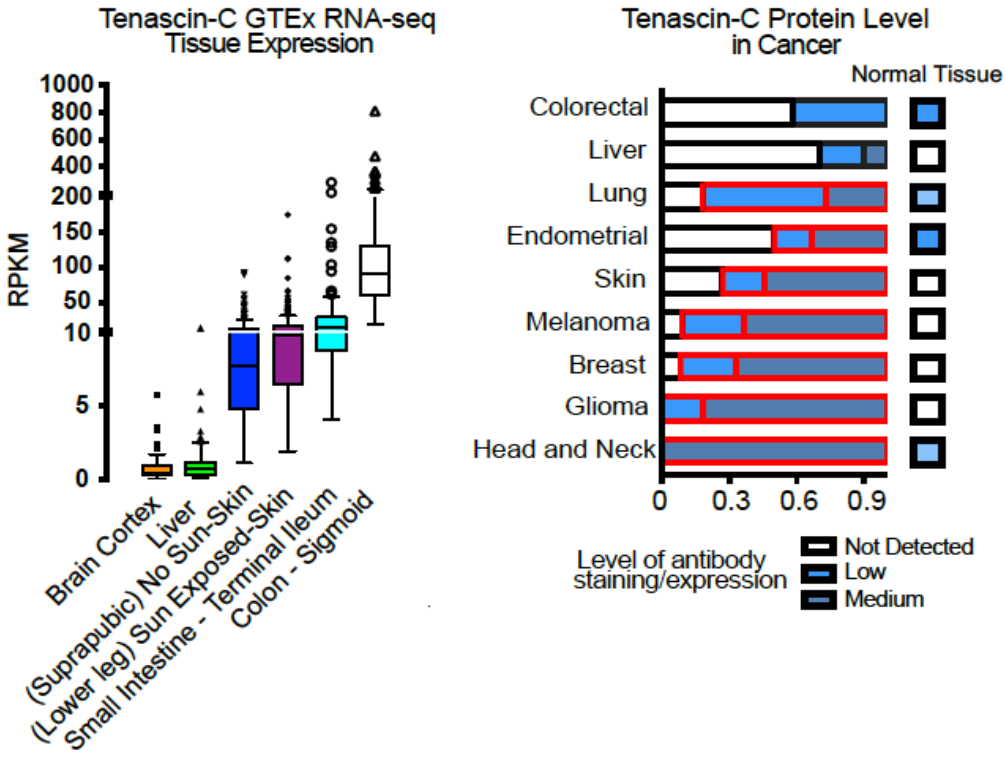

Figure 3: Tumor supportive ECM: TNC expression in normal and malignant tissue

Tenascin (TNC) is largely absent in mature tissue (Left) where expression is tightly regulated. However, TNC expression is markedly upregulated at the protein level in multiple cancers including brain, bone, prostate, intestine, lung, skin and breast (Pas J et al., 2006). Data are reproduced from the Human Protein Atlas (Uhlén M et al., 2015, 2017).

Tenascin-C is largely absent in adult tissues but is paradoxically and aberrantly present in several cancers (**Figure 2**) (**Tucker RP et al., 2009; Uhlén M et al., 2015, 2017; Orend G et al., 2006**). Invasive cancers, including metastatic melanoma have enrichment of TNC at the invasive front. This expression pattern in melanomas is linked to poor recurrence-free survival (**Oskarsson**

T et al., 2011; Lowy CM et al., 2015). Interestingly, TNC expression persists in melanomas, even in the absence of growth factors (**Herlyn M et al. 1991**). Additionally, increased expression of TNC is indicated during the transformation of melanocytes (**Hoek K et al., 2004**). TNC expression continues to increase during progression from benign to metastatic melanoma, indicating a functional importance in metastasis. However, mechanisms governing how melanomas acquire TNC expression are currently ill-defined, especially with regard to dynamics of expression and the correlate function(s).

Our previous studies indicate that the overexpression of TNC-EGFL monomers are sufficient to convert a radially invasive melanoma cell to vertically invade human three-dimensional organotypic skin organ cultures (SOC) (**Grahovac J et al., 2012**). Coupling these findings to evidence that (i) TNC can drive EMT programs in breast cancer (**Nagaharu K et al., 2011**), (ii) activate CAF secretion of TNC (**Chiquet-Ehrismann R et al., 1986; Mackie EJ et al., 1987**), (iii) and promote melanoma progression and resistance to doxorubicin *in vitro* (**Fukunaga-Kalabis M et al., 2010),** identifies TNC as a suspect player in supporting melanoma.

Thus, the tumor and stroma coordinate to develop the TME by reverting to onco-fetal development and wound healing programs. Presentation of ECM, matricellular proteins, and soluble signals in this fashion largely reprograms malignant and stromal cells to promote progression. Overtime, accumulation of this tumor-associated ECM results in invasion and resistance. However, despite experimental correlations to clinical biopsies, the specific mechanisms driving the evolution of tumor-ECM remain elusive. This has prompted broader investigations into the complete repertoire of ECM and how these molecules shift in expression to favor progression.

1.4 The notion of a core matrisome

There is considerable overlap of a large number of 'onco-fetal-wound' ECM reported during malignant progression. These molecules and matricellular proteins are often temporally restricted yet reappear during the establishment of the TME. This led to an effort to characterize the ECM to determine composition and functions in tumors. Genomics provided the key the elucidating specific identifiable features of ECM proteins based on distinctive structures and the repetition of conserved domains (**Engel J et al., 1996; Hohenester E et al., 2002**). The ECM proteins comprise a relatively small repertoire (~100 proteins) of phylogenetically conserved amino acid domains which have been characterized and defined *in silico.* This inventory of proteins, termed the core matrisome (CM) represents ~278 genes for ECM glycoproteins, proteoglycans and collagens with matrisome-associated proteins comprising ~778 genes representing ECM regulators, ECM-associated, and secreted factors (**Naba A et al. 2012**). These genes are differentially expressed and dictated by the contextual or pathological tissues from which they are extracted. The composition of the ECM proteins is reported to shift dynamically in cancer, where metastatic cells elicit changes distinct from low grade malignancy, again suggesting the tumor ECM is constructed via dynamic reciprocity of tumor-stroma signaling.

These early studies laid the foundation for refined studies, wherein subsets of the CM or CM-associated proteins could be further enriched to select targets for specific malignancies. Despite correlative evidence that excessive deposition of ECM is linked to poor prognosis, only incremental advances have been made towards understanding which specific proteins contribute mortality.

Recently, bioinformatics has helped bridge this gap, enabling high level analyses of ECM signatures that are most common between multiple cancer types. These gene-signatures can then

tested against clinical samples to develop a predicative core matrisome (**Yuhzalin AE et al., 2018**). The outcome of this study is a nine-gene ECM signature among solid adenocarcinomas that was shown to have predictive value. This signature contains collagens (e.g. *COL1A1*) as well as secreted molecules which bind ECM (e.g. osteopontin, SPP1). These signatures were also linked to EMT, hypoxia, angiogenesis and inflammation by gene set enrichment analysis thus, providing a biological basis for defining hitherto disparate ECM molecules. A similar approach discovered that cancers with mutations in BRAF, SMAD4 (TGFβ pathway), TP53 and amplifications in MYC activated a TGFβ response in CAFs to produce cancer-ECM and resistance to programmed death-1 (PD1) check point inhibition (**Chakravarthy A et al., 2018**). The high-grade expression of TGFβ in CAFs was predicted to correlate with immune exhaustion and PD1 blockade in patient melanoma samples using the dataset from Tirosh et al., 2016. The study was able to conclude that high predicative scores in CAFs positive for cancer-ECM expression in addition to TGFβ1/ TGFβ2 expression, was indeed powerful enough to predict PD1 blockade ($p<0.007$). Thus, targeting the TGFβ signaling axis may prove therapeutically beneficial in the effort to normalize tumor ECM. This notion is underscored by evidence that despite being 'immunologically hot,' tumors expressing a negatively prognostic ECM signature correlated with the eventual loss of checkpoint inhibition prior to clinical manifestation.

Despite the potential insights offered from defining a core cancer matrisome, these *in silico* approaches do have limitations. For example, these gene signatures are often based on subsets of disease types or small patient populations. The analyses are also computationally derived and based on post-hoc analysis of clinically treated and documented patients. Therefore, there is currently a lack in our understanding for how these molecules interplay upstream of the clinical

manifestation of disease. Here, the gap regarding the mechanistic processes that underly therapy failure and how cancers co-opt the TME to promote invasion and metastasis remains unbridged.

1.5 Hypothesis and major findings

Given the strong association between secreted signals and pathological ECM, this study was developed to **(I)** elucidate the mechanism by which TGFβ drives the phenotypic conversion of CAFs, including how the stroma modifies microenvironmental ECM, and **(II)** Therapeutic strategies to target the TGFβ signaling axis to prevent melanoma metastasis by limiting the inflammation and development of a pro-tumorigenic ECM. This thesis shows that the inflamed TME is characterized by the expression of immature ECM proteins and inflammation. We elucidate that the pathological matrisome produced by αSMA^+ CAFs promotes melanoma invasion and proliferation. Transforming growth factor-β receptor (TGFβR) promotes CAF expansion in response to the secretome of therapy-stressed melanoma. Blockade of TGFβR suppresses CAF inflammation and normalizes the ECM to halt melanoma progression and proliferation. Combined melanoma and stromal therapeutic targeting were exploited and shown to halt melanoma metastasis.

2.0 Materials and Methods

2.1 Cell culture

WM983A, WM983B, WM1158 were purchased from the Wistar Collection at Coriell Cell Repository. Cells were cultured in a mixture of DMEM (Cellgro, 10-014-CV) and Leibovitz L15 (Gibco, 11-415-064) at a ratio of 3 to 1 and supplemented with 5% (v/v) fetal bovine serum (FBS, Gemini Bio, 100-500) and 100 units per milliliter penicillin and streptomycin (Gibco, 15-140-48). A375 melanoma cells were from Dr. John Kirkwood (UPCI), originally obtained from ATCC and cultured in DMEM (Cellgro, 10-013-CV) supplemented with 10% FBS and 100 units per milliliter penicillin and streptomycin. Primary melanoma patient cell line TPF-12-293 melanoma cells were from Dr. John Kirkwood (UPCI) and cultured in RPMI 1640 (Gibco, 11875119) supplemented with 10% (v/v) FBS, 1% Non-essential amino acid (NEAA, Gibco, 11140076), 1% HEPES (Gibco, 15630130) and 100 units per milliliter penicillin and streptomycin. Fibroblasts HS68, TP-1170, and melanoma derived CAFs TP-50236 and TP-50306 (tumor adjacent and tumor distant) were cultured in DMEM (Cellgro, 10-013-CV) 10% (v/v) FBS and 100 units per milliliter penicillin and streptomycin. Cell lines were cultured at 37°C in 5% CO2 and the medium was replaced as required.

2.2 Generation of Therapy Resistant Cell Lines

Vemurafenib and PD153035 resistant melanoma cell lines were developed according the method of Obenauf *et al.* 2015. Human melanomas A375, WM983B and WM1158 were seeded at low density in 5cm tissue cultured treated dishes for 24 hours in normal growth medium. The media was then replaced with either DMEM 5% FBS or normal growth medias supplemented with PD153035 (Selleck Chemicals, S1079) or Vemurafenib (Selleck Chemicals, S1276), respectively. Therapy sensitive cells were exposed to 5μM PD153035 and 1μM for W983B and 3μM Vemurafenib for A375 and WM1158. After 8 weeks of treatment resistant clones $^{(R)}$ of A375^{R}, WM983B^{R} and WM1158^{R} were analyzed for dose-dependent responses to inhibitors and receptor status was analyzed by western blot. Increased expression for EGFR and levels of phospo-ERK and phospo-AKT were compared to the therapy sensitive parental cells (**Figure 19B**).

2.3 Patients and Biospecimens

All experiments using patient tumors were consented and approved by the Allegheny Health Network and are exempt by the University of Pittsburgh IRB. Tumors from melanoma patients were surgically excised and washed in DMEM (Cellgro, 10-013-CV) containing 2(x) antibiotic/antimycotic solution (Sigma, A5955). Tissues were then washed in Hanks balanced salt solution (HBSS, Gibco) containing 1% antibiotic/antimycotic (A/A). Tumors were transferred to a 10cm dish mechanically minced using sterile razor blades to less than 1 sq. mm and incubated win 70units/ml collagenase (Worthington, M2N5857) and 50units/mL Dispase (Gibco, 17105-041) dissolved in serum free DMEM (10-013-CV). Tissues were incubated for 1hr in the

dissociation media at 37°C in 5% CO_2. Next, tissues were mechanically separated using the plunger from a 10ml sterile syringe and incubated for an additional hour at 37°C in 5% CO_2. The enzymatic dissociation media was quenched with normal growth medium containing 20%FBS. The cell solution was strained through a 40μm mesh filter (BD Biosciences) into a 50ml falcon tube. Cells were spun at 1000 r.p.m for 5 minutes, aspirated and resuspended in primary melanoma media (RPMI 1640 (Gibco, 11875119) supplemented with 10% (v/v) FBS, 1% Non-essential amino acid (NEAA, Gibco, 11140076), 1% HEPES (Gibco, 15630130) and 1% penicillin and streptomycin). Cells were allowed to grow for 5 days after which colonies of melanoma were selected and expanded in 12-well plates. Patient matched fibroblasts were also collected and cultured separately in DMEM (Cellgro, 10-013-CV) 10% (v/v) FBS and 1% penicillin and streptomycin. Melanomas were positively identified by gp100 (Abcam, ab137078) or MiTF (ab3201). Fibroblasts were confirmed by morphology and the presence of αSMA (Sigma, A2547 Clone 1A4).

2.4 Cell Cocultures

Cocultures were established by plating fibroblasts in 6 and 12-well plates at 10,500 cells per sq. cm and allowed to adhere overnight in normal growth media. The following day drug sensitive A375 or vemurafenib resistant $A375^R$ cells were seeded into 0.4μm transwell inserts (Falcon, 353090, or 353180) in normal growth media and allowed to adhere overnight. Media was then replaced with low serum (2% FBS) media containing vehicle. 1μM vemurafenib, selumetinib (Selleck Chemicals, S1008), or PD153035, with media for control wells plated at the same time. For experiments with TGFβR antagonists, 1μM vactosertib (Selleck Chemicals, S7530) or 10μM

SB431542 (Selleck Chemicals, S1067) and 1μM infigratinib (Fibroblast growth factor receptor, FGFR, Selleck Chemicals, S2183) diluted in low serum, were added to fibroblasts prior to melanoma seeding. After 72hr, conditioned media was harvested, plates were washed with cold PBS (Cellgro, 21-040-CV). Cells were then prepared for analysis of RNA, protein or immunofluorescence. Coculture experiments were independently performed three times and a representative experiment is shown.

2.5 Conditioned Media Protocol

To generate conditioned media, $1x10^6$ drug sensitive melanoma cells were plated in 10cm dishes in DMEM (Cellgro, 10-013-CV) supplemented with 10% FBS and 1% penicillin and streptomycin and allowed to adhere overnight. The media was then replaced with low serum (2% FBS) media containing vehicle, 1μM vemurafenib, 0.1μM selumetinib and 5μM PD153035 (1μM for WM983B) with control wells plated at the same time. After 72hr cells were approximately ~90% confluent and media was collected, centrifuged at 1,500 r.p.m. for 5 minutes, filtered and aliquots were stored at -80°C. Media was used for key coculture experiments.

2.6 Quantitative PCR

Whole RNA was isolated from treated melanoma cells using the RNAeasy Mini Kit (Qiagen) according to manufacturer specifications. RNA from coculture experiments was isolated using Trizol (Ambion) separately from fibroblasts and melanomas after transwells were put into a

fresh 6-well plate RNA was reconstituted in nuclease-free water and concentration was measured by Nanodrop. Samples were stored at -80°C prior to reverse transcription to derive cDNA using the QuantiTect reverse transcription kit (Qiagen, 205313). Samples were stored at -20°C. Quantitative PCR was performed using RT^2 SYBR Green Master mix (Qiagen) in 96 well plates on a Stratagene Mx3000P. Quantification was calculated using the ΔΔCt method with GAPDH as the control. All data points represent at least 3 technical replicates and experiments were independently performed twice. A representative is shown.

2.7 Immunoblotting

Cells were rinsed in PBS and lysed using ice cold RIPA buffer containing protease inhibitor cocktail (Calbiochem, 535140) and phosphatase inhibitor cocktail (Sigma, P5726) according to manufacturer specifications. For the analysis of matrikines or extracellular matrix proteins, cells were washed with PBS and lysed directly into laemelli buffer containing 10% β-mercaptoethanol. At this point all samples were sonicated on ice. Samples were then placed at 4°C for 20 minutes and then centrifuged at 15,000 r.p.m. for 30 minutes. Protein concentrations of RIPA samples were determined using a BCA Protein Assay Kit (Pierce). In most cases samples were collected using both buffers and concentrations were assumed to be similar based on protein weight. Proteins were separated by SDS-PAGE using bis-tris 7.5% polyacrylamide gels in tris-buffered saline and transferred to PVDF membranes using a Bio-Rad transfer system according to standard protocols. Membranes were blotted with primary antibodies diluted in 5% bovine serum albumin in tris buffered saline 0.1% Tween 20 and 0.02% sodium azide (TBST) for Tenascin C (ab108930), MiTF (ab3201) from Abcam, E-cadherin (3195), Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)

(4370), Pan AKT (4685), GAPDH (5174), tEKR (p44/42 MAPK (Erk1/2)) (9102), α-Tubulin (2144) from Cell signaling, and αSMA (A5228) from Sigma Aldrich. All primary incubations were on a low speed shaker overnight at 4°C. Membranes were incubated with secondary antibodies from Sigma Aldrich 12-348 (Rabbit) and 12-349 (Mouse) diluted in 5% non-fat dried milk diluted in TBST for 2hr. All antibodies are described in the Supplementary Information. Membranes were washed three times with TBS in between antibody incubations and detected using chemiluminescence according to standard procedures. Radiographs were processed and scanned.

2.8 Immunofluorescence and Imaging

Cells were seeded onto glass coverslips at $5x10^5$ per well in 12-well plates in normal growth media and incubated overnight. After coculture or drug treatment cells were washed in PBS and fixed in 4% paraformaldehyde. Cells were lysed using 0.1% Triton-X 100 (Fisher Scientific) for 15 minutes on a rocker at medium speed. Samples were then blocked for 1hr using 5% BSA diluted in PBS. Samples were then washed and incubated with primary antibodies in 3% BSA /PBS overnight at 4°C. Cells were washed with PBS and incubated in 1% BSA/PBS with the species appropriate secondary antibody (Supplemental Information). For F actin staining, 100ng/mL AlexaFluor™ 594 or 488-AlexaFluor™ phalloidin (Thermo Fisher) was diluted in 1% BSA/PBS with secondary antibodies and incubated for 1hr. Samples were then washed in PBS and incubated in 6.6μM Hoechst 33342 (Thermo Fisher) in 1% BSA/PBS, washed in PBS, and mounted in Prolong gold antifade reagent (Invitrogen). Epifluorescence was performed on Olympus X71 microscope equipped with a Spot Insight™ CCD digital camera (Spot Imaging

Solutions) using 10, 20 and 40x/0.6na objective (Olympus). Image analysis was performed in post using ImageJ (https://imagej.nih.gov/ij/) or NIS Elements Advanced Research software.

2.9 Immunohistochemistry

Patient samples were collected under standard clinical management with approval of the UPMC institutional review board for protocol(s) 1611. Tumor samples from the Allegheny Health Network were considered exempt. Patient-informed consent was obtained for the research performed in this study. Human melanoma microarrays ME1004a, ME1004g, and ME1004h were purchased from Biomax. Xenograft tumors were collected from euthanized animals in fresh 10% neutral buffered formalin and paraffin embedded (FFPE) through a series of dehydration steps at the University of Pittsburgh Biospecimen Core. Tumors were then sectioned using a sliding microtome (Fisher). Tumor slices (5μm) and tissue arrays were de-paraffinized by baking for 1hr at 55°C, then immediately placed into Xylene (Fisher) baths (3 each), transferred to 100%, 95% and 80% ethanol. Following, this samples were washed in PBS five times and placed into citrate buffer at 95°C for 10 minutes and cooled to RT for 1.5hr. Samples were then washed in PBS and quenched with 3% hydrogen peroxide. After PBS washing, samples were blocked in 20% goat serum for 1hr, and primary antibodies were diluted in blocking buffer for overnight incubations at 4°C. Samples were stained with of αSMA (Sigma, A2547 Clone 1A4), Tenascin (BC-24, Santa Cruz Bio.), Decorin (R&D), and Collagen I (Abcam). Samples were then washed with PBS and incubated in species appropriate secondary antibodies. All antibodies are detailed in the Supplementary Information. After incubation, samples were treated with ABC reagent (Vector laboratories, PK-6100) for 30 minutes, washed in PBS and developed with DAB reagent kit

(Vector laboratories, SK-4100), counterstained with H&E, clarified (Richard Alan Scientific) and dehydrated in ethanol and xylene. Samples were mounted (Fisher Scientific, SP15-500) and imaged on an Olympus BX40 upright microscope equipped with a Spot Flex^{TM} CCD digital camera (Spot Imaging Solutions) using 4x, 10, 20x and 40x (UPlan) objectives (Olympus). Image analysis was performed using ImageJ.

2.10 Lentiviral Infection

All constructs are described the supplementary information. Fluorescent reporters for GFP and RFP were purchased from Origene and added to normal cell growth medium containing polybrene (6μg/mL). A total of 5-10μL or viral particles was added to individual 96 wells containing melanoma cells lines that were plated the day before at 2000 cells per well. Transduction and fluorescence were confirmed after 36 hours and cells were split to 12-well plates for expansion. Clones were selected using puromycin and blasticidin, respectively. Fluorescence clones were expanded in normal growth media containing antibiotic and further purified by FACS at the University of Pittsburgh Flow Cytometry Core and were used for spheroid studies.

2.11 Three-dimensional Spheroid Assay

The method of Kaur et al. was followed for the development of assays (Supplementary Ref. 3). Briefly, sterile 96 well plates were coated with 50μL of 1.5% agarose (Sigma, A9539) dissolved in DMEM (10-013-CV) that was heated to a boil. Melanoma cells were then plated at

$1x10^4$ cells diluted in normal growth media at 50µl per well. After 72 hours cells formed dense tissue spheroids. For spheroids and fibroblast cocultures, $4x10^3$ fibroblasts were seeded into 8µm falcon permeable supports – transwells, (Falcon, 353097) into 24-well plates and adhered overnight. Otherwise, melanoma spheroids were harvested using a p200 micropipette and deposited into 50µL of 2 or 4mg/mL rat tail collagen type I (Corning, 345249) dissolved in 50mM HEPES pH 8. Normal growth media with or without inhibitors was added to the upper and lower chamber of the transwell. Quantitation of invasive outgrowth was performed using ImageJ software.

2.12 Thick collagen invasion assays

Acellular collagen I matrices were prepared using rat tail collagen type I (Corning, 345249) at 3mg/mL and casted into 8µm transwells (Falcon, 353097) 50µl per insert. For studies involving matrix proteins, 4µg/ml of tenascin-C (Millipore, CC065) or decorin (R&D Systems, 143-DE-100) were added prior to gel neutralization. Matrices were placed into 37°C in 5% CO_2 to polymerize. Melanoma cells were seeded into the scaffolds at $5x10^4$ in low FBS media and with normal growth media in the lower chamber. Gels were fixed after 72hr and stained as described with a modification for phalloidin (Hopper et al., 2006). Briefly, collagen inserts were washed with PBS, fixed for 30 minutes in 4% formaldehyde-0.25% glutaraldehyde in PBS, treated with 0.1% Triton X for 30 min on ice, and stained with 0.2µg/ml 488- AlexaFluor™ phalloidin (Molecular Probes, Invitrogen) and 2µg/ml DAPI (Sigma) in dark for 3h. Gels were imaged directly in the inserts in PBS on the Nikon Swept field Confocal Microscope (TSI inverted) using 10X and 60X 1.4 NA objectives. 3D volume representations of Z-stacks were made using Nikon Elements Advanced

Research software and surfaces of the invading cells were modeled with Imaris software based on the absolute intensity of phalloidin staining with 3μm surface area detail level (Bitplane, Zurich, Switzerland). Quantification of the depth of invasion was performed with the length measurement tool of Nikon Elements Advanced Research software by measuring 60 random positions per sample from the surface of seeded cells to the bottom most detected intensity. Experiments were repeated three times.

2.13 Organotypic three-dimensional skin reconstructs

Skin organ cultures were established as previously described (Grahovac *et al.*, 2012; Simpson *et al.*, 2010), with modifications. Briefly, $15x10^4$ primary human fibroblasts were seeded in 1.5 ml 2mg/ml collagen gels alone or with the addition of 4μg/ml of TNC, DCN or both, in 24 well plates. After 5 days in submerged culture, $25x10^4$ normal human keratinocytes were seeded on top of contracted gels. Keratinocytes were allowed to adhere and 48h later $10x10^4$ WM1158 cells were seeded in the center of the plug within the 4,7mm R cloning rings (Bel Art, 14-512-78, Thermo Fisher Scientific, Pittsburgh, PA), let attach for 8h and then the cloning rings were removed. After 3 days, the cultures were lifted to the air liquid interface to allow epidermis maturation and the medium was replenished every other day for total of 20 days of culture. The samples were fixed and paraffin embedded and H&E stained as previously described (Hood et al., 2010). The experiment was repeated two times with similar results.

2.14 Animal Studies

All animal studies and procedures were approved and performed at the AAALC-accredited IACUC of the Veteran's Administration Pittsburgh Health System. Adult (5-6-week-old) NOD-scid IL2Rg^{null} (05557, Jackson Laboratory) female mice were anesthetized with ketamine/xylazine and buprenorphine to relieve pain. Spleens were exposed by left lateral incision in a sterile tissue culture hood wherein 5 x10^5 human A375 or patient metastatic 12-293 melanomas, and 2.5 x10^5 metastatic WM1158 cells were injected at 50μL per animal using a 26.5-gauge needle. Incisions were closed using 5-0 vicryl suture and two staples. Animals were warmed and monitored until recovery and housed individually. Tumors were allowed to grow untreated for 4 weeks be euthanasia. For therapeutic studies, animals bearing 12-293 tumors were injected on Day 7 with vehicle (10% DMSO in FBS) or MEK inhibitor (AZD6244, 10mg/kg) or a combination of AZD6244 and vactosertib (5mg/kg) prepared fresh in warmed FBS. On day 30 animals were euthanized to collect spleen, liver and lungs for micro-or-macrometastatic analysis.

Patient derived xenograft (PDX) studies were conducted using patient biopsies received from the Allegheny Health Network and were approved by both institutions. Fresh excess tissues were surgically removed from patients with treatment refractory melanomas and saved for pathology and analysis, while small 10mm^3 pieces were washed in DMEM (10-013-CV) with 2x (A/A) and stored in cold HBSS with 1x (A/A). Five-week-old NOD-scid IL2Rg^{null} were anesthetized and treated as treated as previously described. A small pocket in the left flank was formed for subdermal placement of tumors. The incisions were sutured, stapled, and animals were warmed and allowed to recover before individual housing. PDX tumors were grown in P0 mice for approximately 3-6 months as determined by tumor size (~100mm^3) using the formula, tumor volume = (D x d)^2/2 in which D and d represent x and y tumor diameter. These tumors were split

into ~50mm^3 sections for analysis and cell culture, and passage to a P1 NOD-scid IL2Rg^{null} mouse. Animals in the P1 cohort were euthanized after ~6 months and tumor, liver, spleen and lung were harvested for analysis. De-identified information of tumors is described in the Supplementary Information.

2.15 Statistical Analysis

Data are presented as the mean ± s.e.m., for three independent experiments. Significance between comparisons with two independent variables was determined by a Student's two-tailed unequal variance *t*-test. One-way ANOVA was used to compare significance for more than 2 variables and between biological replicates. Significance levels and the tests used to determine these values are reported in the figure legends. Significance is represented in all figures with symbols denoting *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$

3.0 The Therapeutically Inflamed TME Drives Melanoma Progression

3.1 Introduction

Therapeutic regression of the tumor microenvironment (TME) is often hampered by drug resistance **(Holohan C et al., 2013)**. This inevitable feature is common in solid tumors which contain diverse tumor and cancer-supportive stromal populations. Together, these cells secrete heterotypic signals to sustain tumor proliferation, invasion, and drive treatment attenuation (**Straussman R et al., 2012; Junttila MR et al., 2013; Egebald M et al., 2010**). Treatment failure is complex and linked to multiple paradigms that evolve with the TME. For example, prolonged exposure to inhibitors of $BRAF^{V600E}$, a mutation present in ~35-50% of melanomas, drives the formation of resistant clones which are supported by a drug-sensitive cell secretome (**Cancer Genome Atlas Network., 2015; Villanueva J et al., 2010**). This therapy-induced secretome is reported to promote tumor progression in melanoma and is associated with the loss of disease stabilization within 6-12 months (**Obenauf et al., 2015; Shi H et al., 2014**). Dual inhibition of RAF and PI(3)K/AKT/mTOR was found to be effective in treating $BRAF^{V600E}$ resistant melanomas, however combined therapy preceding checkpoint inhibition failed to prevent emergence of metastatic disease due to MAP2K activation (**Richmond CS et al., 2019**). Single cell profiling of tumors from melanoma patients has revealed complex cellular ecosystems wherein a high density of cancer-associated fibroblast (CAFs) are associated with melanomas expressing a genetic signature for BRAF/MEK resistance. These same CAFs lose the expression of a unique set of immune modulating genes upon removal from the TME implying that these phenotypes are context dependent (**Tirosh et al., 2016**).

The TME is a site of persistent inflammation due to the reactive stroma, which is associated with angiotropism in melanoma (**Bald T et al., 2014**). Among the infiltrating immune cells, CAFs are pro-angiogenic, pro-inflammatory, produce immune suppressive cytokines, and aberrantly deposit extracellular matrix (ECM) (**Kim DJ et al., 2018; Quail DF et al., 2013**). CAFs lack a clear molecular distinction in comparison to their normal counterparts but differ in the expression of α-smooth muscle actin (αSMA), S100A4 and type 1 collagen (COL-1) (**Kullari R., 2016; Sugimoto H et al., 2006**). In melanoma, the production of ECM proteins is significantly altered compared to normal skin; components characteristic of an immature inflamed dermis such as tenascin-C (TNC), fibronectin and secreted protein acidic and cysteine rich (SPARC) are upregulated, while mature matrix components such as decorin (DCN) and certain collagens are down-regulated (**Ilomen S et al., 2004; Pickup M et al., 2014; Kunigal S et al., 2006; Hood BL et al., 2010**). We have previously reported that invading melanomas secrete TNC at the invasive front (**Grahovac J et al., 2013**). Separately, invasive breast micrometastases expressing TNC displayed enhanced survival and colonization (**Oskarsson et al., 2011**). Taken together, changes in ECM and links to poor prognosis in melanoma illuminate the matrix as a vital component for progression. However, large inflamed tumors are typically necrotic and inactive in comparison to the invasive front and adjacent inflamed stroma.

Prompted by this, we examined the therapy induced secretome to uncover signals that promote CAFs to produce aberrant ECM. Transcriptional profiling of drug-stressed melanoma revealed increases in pro-inflammatory cytokines including transforming growth factor-β (TGFβ). The presence of TGFβ in cell culture resulted in the expression of pathological ECM in CAFs. Invasive melanomas were more aggressive in coculture with CAFs which was blunted by inhibition of MEK. However, MEK inhibition in cocultures was associated with αSMA^{+} CAF

inflammation. Here, we demonstrate that inhibition of TGFβ receptor I (TGFβRI/ALK4/5) diminishes αSMA$^+$ CAFs and limits metastasis of melanoma *in vivo* by preventing the induction of a therapy inflamed matrisome.

3.2 Results

3.2.1 Tumor microenvironmental matrix is altered during invasion and progression

Invasive melanomas have been reported to occur within an altered dermal environment with features reminiscent of tissue injury or chronic inflammation (**Bald T et al., 2014; Midwood KS et al., 2009**). In these settings, re-expression of TNC provides for both the survival and migration of adherent cells while signaling suppressive small leucine rich proteoglycans (SLRP) are downregulated. Based on our report that inflammatory ECM is redistributed in three-dimensional organotypic skin during melanoma progression, selected ECM protein expression was analyzed in human melanoma tumors (**Hood BL et al., 2010**). Tenascin-C was largely absent in benign nevi which is expected given that expression is typically limited to sites of wound repair and is otherwise absent in adult tissues (**Shao H et al., 2015**). Conversely, SLRP decorin (DCN) exhibits mutually exclusive patterns of high expression where TNC is absent (**Figure 4A**). Interestingly, DCN decreases in expression ($p = 0.0001$, benign nevi vs. lymph node and stage II primary tumors), while TNC increases as melanomas become more metastatic ($p = 0.05$, benign nevi vs. all conditions) (**Figure 4B**). The increase is expected as given that TNC promotes invasion. In fact, TNC increases *in vitro* with metastatic cells producing the highest amounts

(Figure 5A). Melanomas also increase epidermal growth factor receptor (EGFR) and lose epithelial E-cadherin (ECAD) and microphthalmia-associated transcription factor (MITF) as they

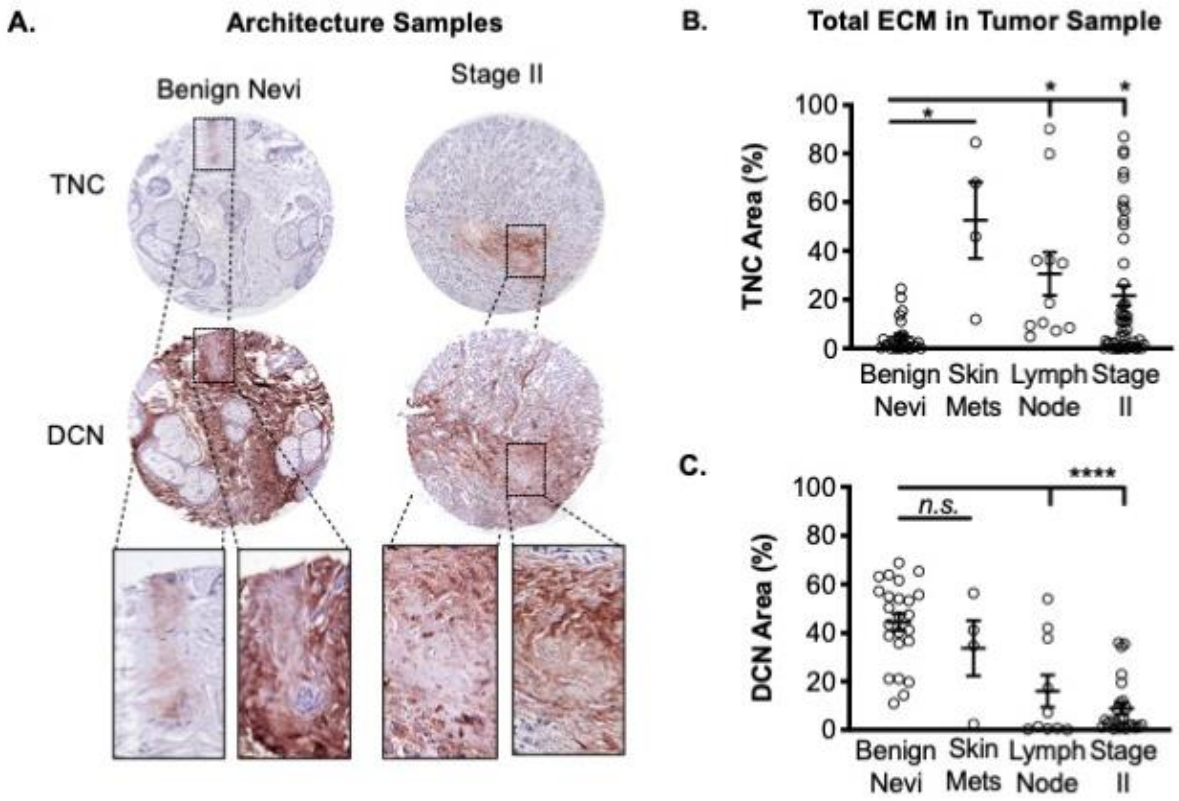

Figure 4: The expression of ECM shifts to promote melanoma progression

(A) Human melanomas drive the remodeling of the extracellular matrix through increased expression of pro-inflammatory tenascin-C (TNC) and progressive reduction of decorin (DCN). **(B)** Quantification of total TNC and DCN expression human melanoma tissue biopsies. Data are presented at means ± s.e.m.; student's unpaired t-test (original magnification 4x). A representative image is shown for selected conditions.

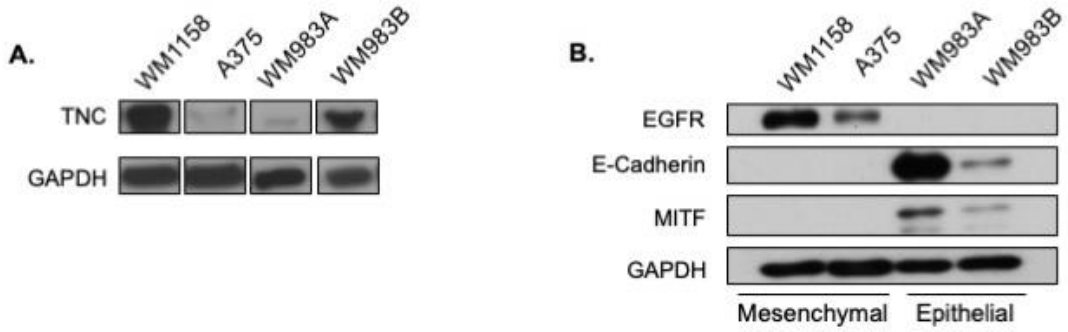

Figure 5: Melanoma phenotype shifts to promote invasion

(A) Western analysis of matrix bound tenascin-C (TNC) from representative melanoma cell lines. **(B)** Analysis of epithelial to mesenchymal (EMT) markers correspond to melanoma phenotype with expression of E-Cadherin and MITF (markers of primary melanomas) and EGFR and TNC expression (markers of metastatic melanomas).

become more mesenchymal and motile during EMT (**Figure 5B**). Malignant melanomas gain expression of AXL kinase which associated with BRAF/MEK resistance and tumor supportive CAF recruitment (Tirsoh et al., 2016). However, we were unable to detect an *in vitro* increase in AXL in metastatic cells (data not shown).

Based on reports that CAFs play essential roles in promoting melanoma outgrowth by limiting drug penetration due to excessive deposition of fibrotic ECM, we assessed whether the tumor stroma was activated in human tissue microarrays (**Lu P et al., 2012; Kim DJ et al., 2018**). We observed an increase in total αSMA^+ which tracked with malignant severity and appears more organized as stress fibers at high-grade, indicative of myofibroblasts (**Figure 6A**). Increased αSMA^+ correlates with a reported increase in CAFs in high-grade serous ovarian cancer (**Pearce OMT et al., 2018**). Collagen type-1 expression was more ubiquitous with respect to the grade of melanoma but tracks with the expression of TNC and αSMA. Thus, increases in fibrotic ECM

correlate with high-grade malignancies that are hallmarked by TNC melanoma invasion (**Figure 6B**). Prompted by the trends from the human tissue microarray, we sought to determine how TNC is distributed *in vivo* with respect to inflamed stroma as TNC has been shown to activate pro-inflammatory tissue destruction in arthritic disease (**Rhumann et al., 2012; Bhattacharyya S et al., 2016**).

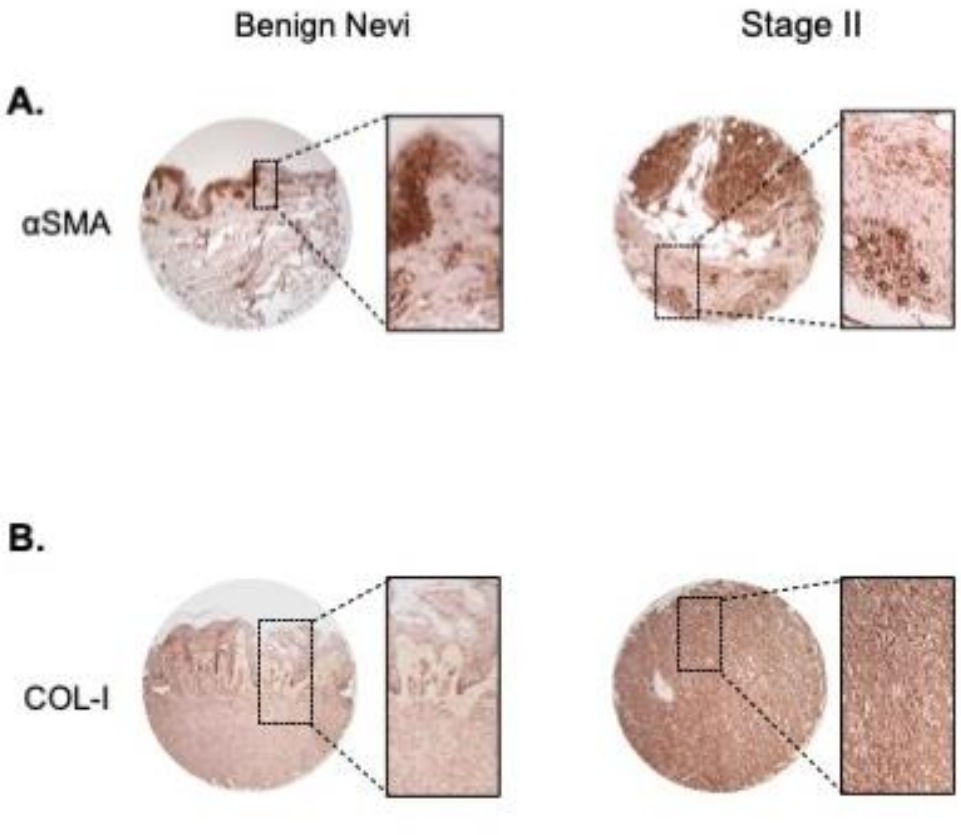

Figure 6: The tumor microenvironment becomes inflamed and fibrotic

As melanoma progresses from benign nevi to stage II the skin dermis increases expression of α-smooth muscle actin (SMA) **(B)** which corresponds to increases in collagen-1 (COL-1) as tumors become inflammatory and fibrotic.

To model the progression of invading melanomas *in vivo*, we performed intrasplenic injections of human melanoma cell lines A375 and WM1158, and 12-293 – a patient derived line from a vemurafenib treated brain mass **(Figure 7A**). After splenic tumors were established,

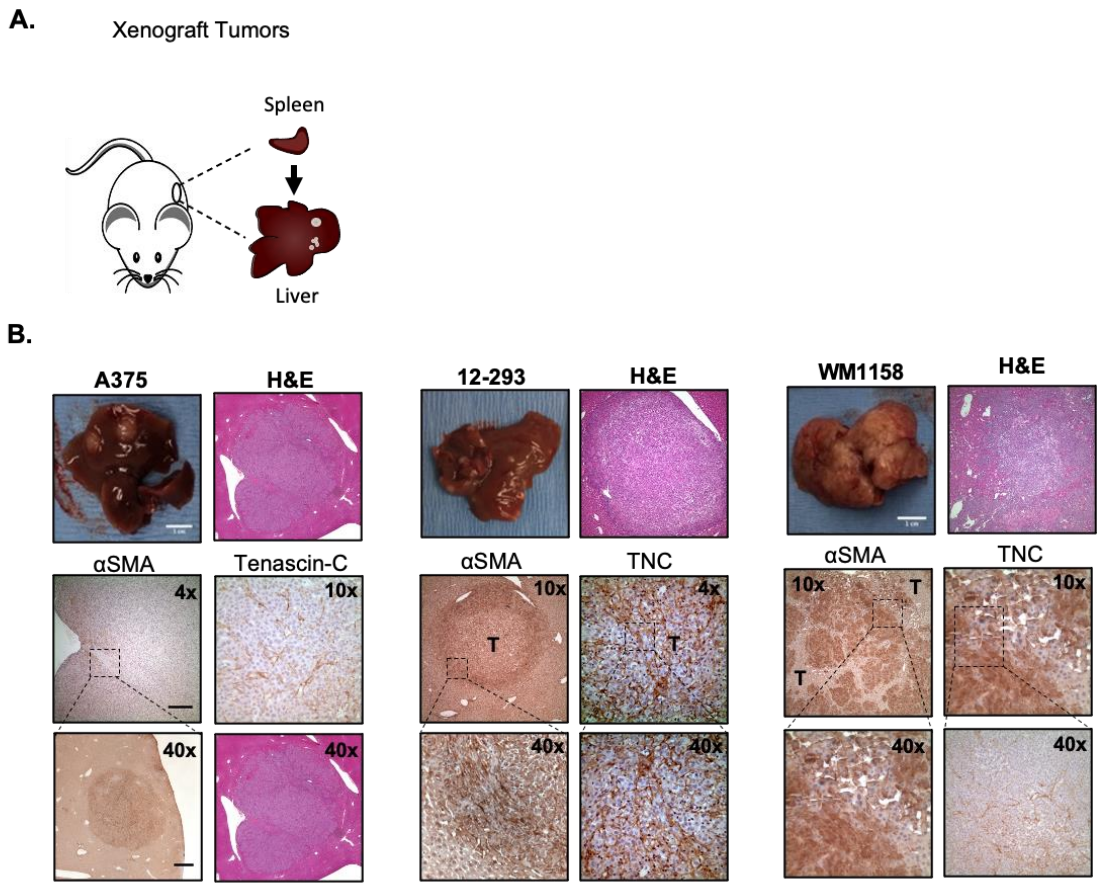

Figure 7: Melanoma progression is promoted by the tumor and the stroma

(A) Schematic and timeline of human xenograft tumor model; spontaneous metastasis from spleen to liver. **(B)** Immunostaining of mouse metastatic liver site for co-expression of αSMA and Tenascin-C. A total of 2 or 3 animals were used for each cell line. A representative image is shown for each condition. Original magnification is labeled in the upper right corner.

animals were monitored and analyzed at 4 weeks. Analysis of metastatic liver nodules revealed co-expression of TNC within the invasive borders and αSMA adjacent to tumor lesions. The amount of TNC and αSMA corresponded to the metastatic potential of the cell line. Thus, A375 tumors show minimal TNC expression in comparison to metastatic 12-293 and WM1158. The metastatic lines also have more peri-tumor αSMA expression whereas A375 nodules appear higher in αSMA than the surrounding tissue at the macroscopic level (**Figure 7B**). Conversely, despite total tumor burden and organ involvment in the spleens of these animals, the distribution of αSMA is less clear for each of the three cell lines (**Figure 8**).

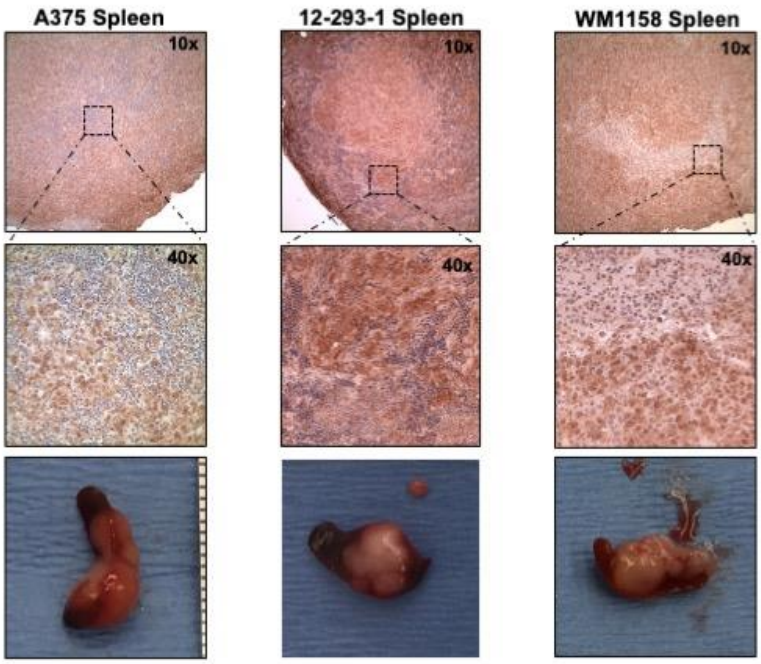

Figure 8: ECM remodeling in the primary site

Immunostaining of mouse spleen injection site does not show the coordinated expression of αSMA that is associated with micrometastatic outgrowth. A total of 2 or 3 animals were used for each cell line. A representative image is shown for each condition.

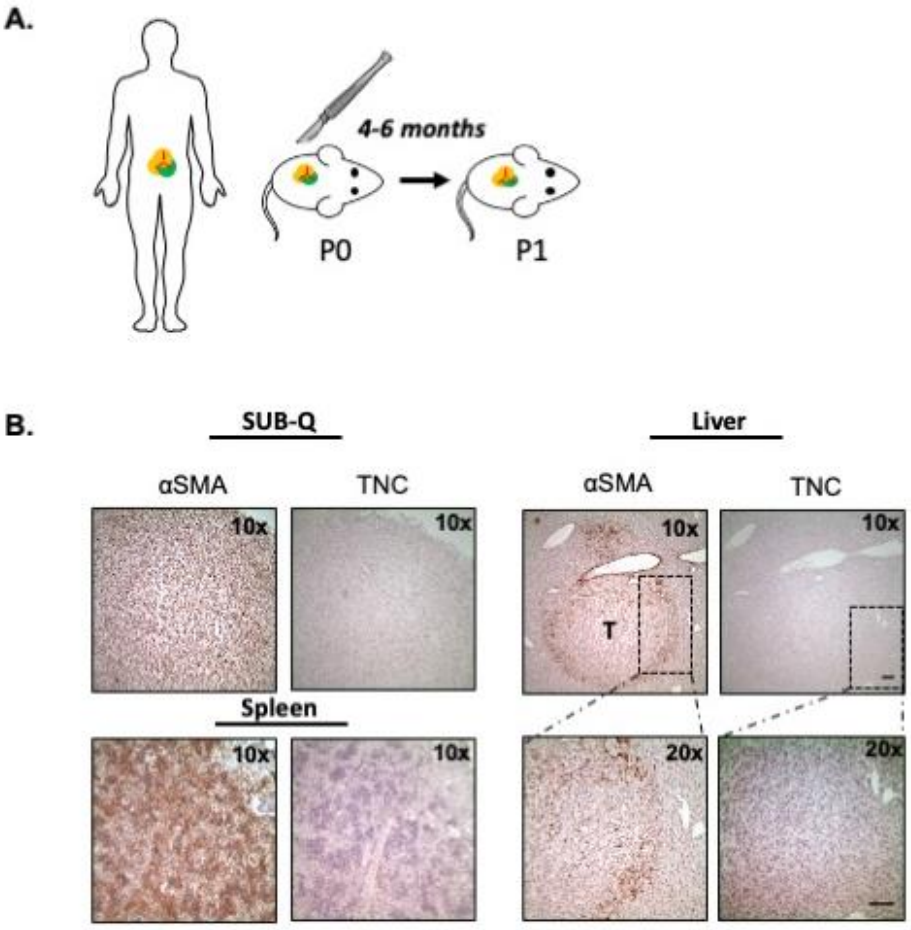

Figure 9: Patient progression is promoted by the tumor and the stroma

(A) Schematic and timeline of patient derived xenograft tumor model **(B)** Immunostaining of human melanoma (SUB-Q) and mouse metastatic sites (Spleen and Liver) for expression of αSMA and Tenascin-C. A total of 2 or 3 animals were used for each tumor. A representative experiment and image are shown for each condition.

As the spleen is not a true representation of the 'primary' site for melanoma, we asked whether TNC and αSMA expression are microenvironmentally regulated based on tumor location. Thus, human patient derived xenograft (PDX) melanoma tumors were used to examine microenvironmental impact on ECM expression. A treatment refractory programmed death (PD1) treated tumor was implanted into the subcutaneous left flank of a NOD-SCID mouse. Subcutaneous tumors were removed after reaching 100mm^3 and passaged to P1 animals where

outgrowth was evaluated for 3-6 months (**Figure 9A, above**). The PDX subcutaneous tumor is positive for αSMA while TNC expression was undetected, in keeping with findings from early stage human melanomas. Metastasis to the spleen shows evidence of architectural remodeling marked by the presence of αSMA and with minimal amounts of TNC, similar to the A375 tumor of the spleen (**Figure 7B**). However, the architecture of the metastatic liver site shows clear evidence of the mutually exclusive pattern of melanoma TNC and αSMA^+ CAFs. The αSMA^+ CAFs colocalize with intratumoral TNC at the invasive front of the nodule. In addition to this, αSMA^+ CAFs appear striatal around the peri-tumor tissue with fibrillar αSMA; these structures are composed of actin stress fibers at the microscale. Expression of actin stress fibers are a hallmark of myofibroblasts.

Thus, despite tumor subtype or ontogeny (cell lines vs. PDX), the mechanisms governing architectural remodeling of ECM appears distinct to the local tumor microenvironment. Given that both melanomas and CAFs increase expression of secreted TNC *in vitro* over time **(Figure 10A-B),** we sought to determine (i) what pro-inflammatory signals were upregulated in melanoma (**ii**) whether inflamed melanomas can activate CAF transformation *in vitro*.

3.2.2 Therapy stressed melanomas inflame the stroma

The tumor microenvironment is a complex niche that is replete with multiple cytokines such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), macrophage chemotactic protein-1 (MCP1/CCL2), and TGFβ, all of which are known activators of CAFs (**Quail DF et al., 2013; Kullari R., 2016**). Based on this, we queried an RNAseq dataset by (GEO accession GSE64671) to ascertain how cytokine expression shifts during therapy-induced stress

of melanomas (**Obenauf et al., 2015**). From this dataset, we detected increased expression of MCP1 (33.57-fold), multiple TGFβ ligands (TGFβ2, 7.66-fold), TGFβ receptors and latent TGFβ

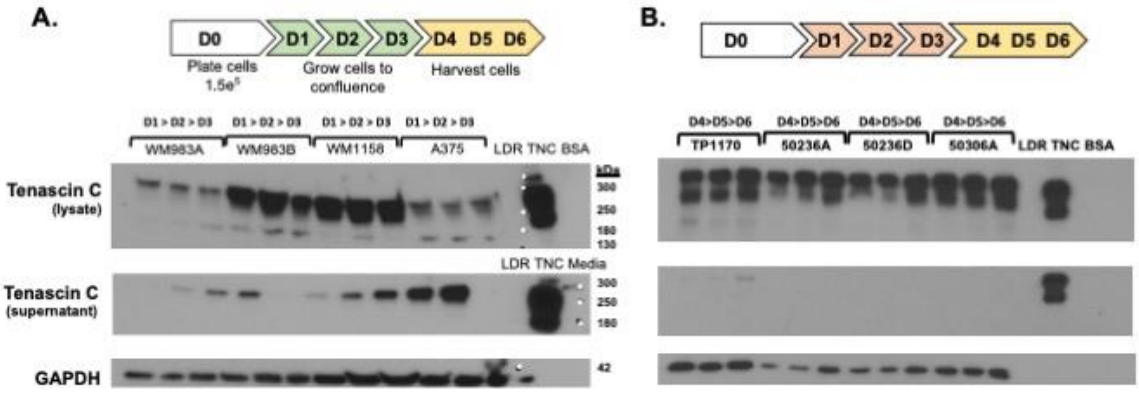

Figure 10: Melanomas and CAFs secrete inflammatory matrix protein TNC
(A) Melanomas increase TNC secretion correlating to metastatic potential where invasive WM983B $BRAF^{V600E}$ show marked induction compared to non-transformed WM983A. Highly metastatic WM1158 secrete the most TNC, while malignant A375 show steady levels**. (B)** The pathological expression of TNC increases with metastatic potential and is maintained in CAFs (Aged – 50236 and Young 50306 Adjacent (A) and Distant (D)) relative to normal human foreskin fibroblasts (TP1170) when comparing the total amount of loaded protein. Samples were directly lysed in laemelli buffer.

transcripts (**Figure 11A**). Jun proto-oncogene (JUN) was induced 7.85-fold at 48hr treatment. Internal and external stimuli trigger c-(JUN) N-terminal kinase (JNK)/ stress-activated protein kinase (SAPK) which include growth factors, cytokines, stress-inducing signals from pathogens, and drugs (**Wagner EF et al., 2009; Hotamisligil GS et al., 2016**). Upon activation, c-JUN forms homo-or-heterodimers with members of proto-oncogene (FOS) and (MAF) families which form transcription activator protein (AP1) (**Eferl R et al., 2003**). Osteopontin (SPP1) was also induced while the leucine zipper protein FOSL1 (FRA-1) was suppressed by vemurafenib stress, in keeping with the report (**Obenauf et al., 2015**). Interestingly, 48hr vemurafenib treatment induced pro-epithelial ECAD and MITF, while AXL and DCN induction were static. However, TNC was

induced nearly 3.87-fold by BRAF inhibition. Prompted by this complex transcriptional network, ingenuity pathway analysis (IPA) was then employed to analyze this enriched gene set by Z-score. Pathways showed downregulation of cell proliferation and increases in scores for cytostasis, innate recruitment, and organ inflammation (**Figure 11B**).

The *in vitro* drug-stress assay was utilized to independently confirm the transcriptional effects of drug-induced cell stress that were obtained from the published dataset (**Figure 12A**). Inhibition of $BRAF^{V600E}$ (vemurafenib, IC_{50} ~30nM) led to an increase in CCL2, TGFβ2 and TGFβR2 in keeping with the dataset (**Figure 11A**). We expanded this analysis by targeting the EGFR pathway and observed EGFR inhibition (PD153035) leads to the transcriptional activation of these genes while downstream inhibition of MEK1/2 (selumetinib, (IC_{50} ~10nM) appears to bypass TGFβ and MCP1 gene expression despite a 10-fold lower dose. This inhibitory MEK1/2 effect is maintained in cell culture where selumetinib induces a phenotypic response at 0.1μM, while comparable doses of vemurafenib and PD153035 have minimal phenotypic effect and do not suppress proliferation (data not shown).

The preliminary evidence from the drug stress assay points to melanoma release of a pro-inflammatory secretome, while Obenauf et al., 2015, reported ~2-fold increase in TGFβ2 and ~5-fold increase in MCP1 using 0.1μM vemurafenib. Thus, given that the results from the drug-stress assay trend, and reports that melanomas release TGFβ with rounding, metastatic potential, and invasion both *in vitro* and *in vivo* (**Cantelli G et al., 2015**), we sought to determine whether CAFs were responsive to therapies and to drug-stressed melanoma in coculture.

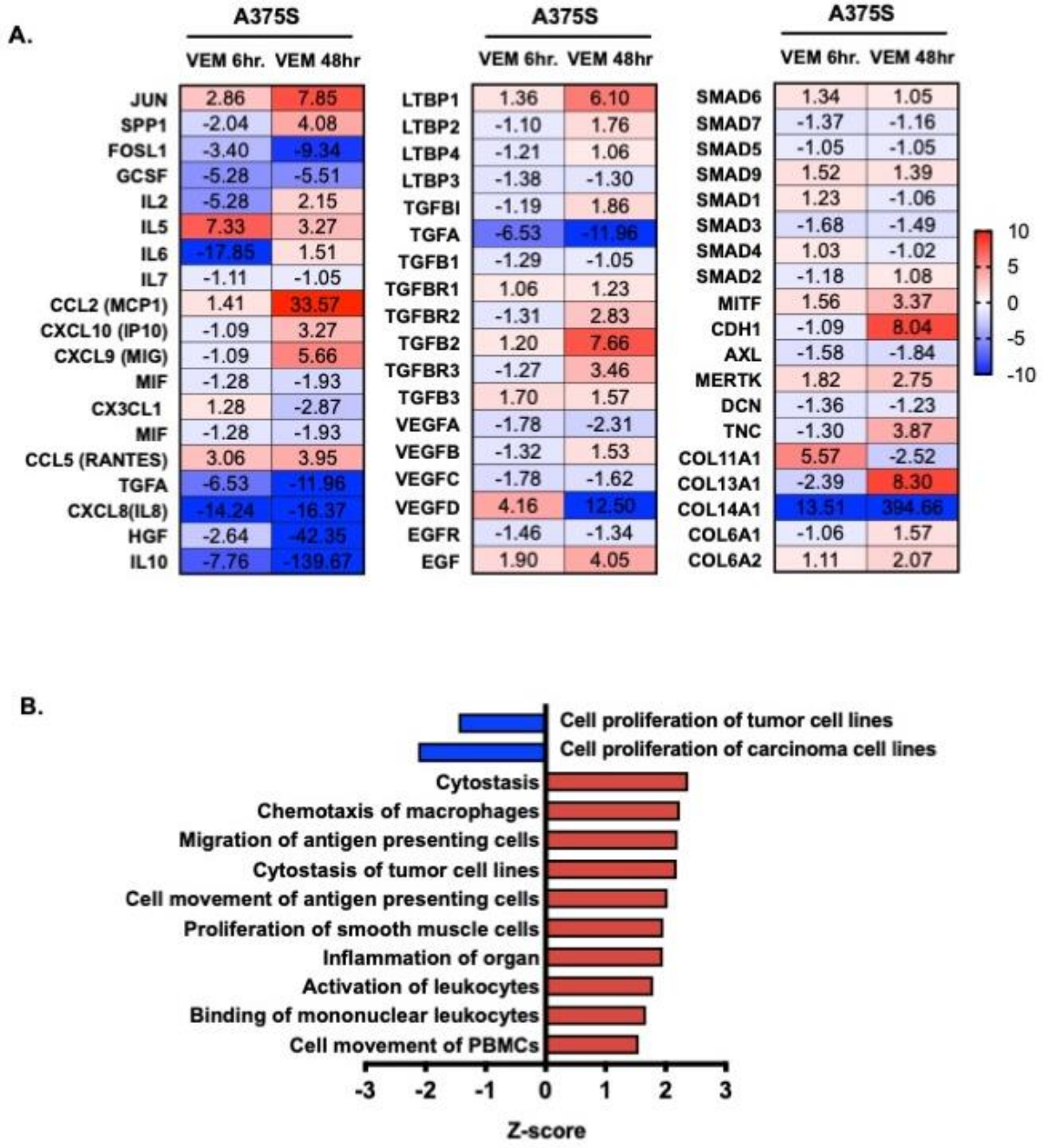

Figure 11: Analysis of therapy induced secretome

(A) Heat map showing log fold change of gene induction after 6 and 48hr of treatment from Obenauf *et al.* 2015 (GEO: GSE64671). **(B)** Ingenuity pathway analysis of selected gene set shows suppression of tumor proliferation with transcriptional increases correlating to innate inflammation and stromal activation.

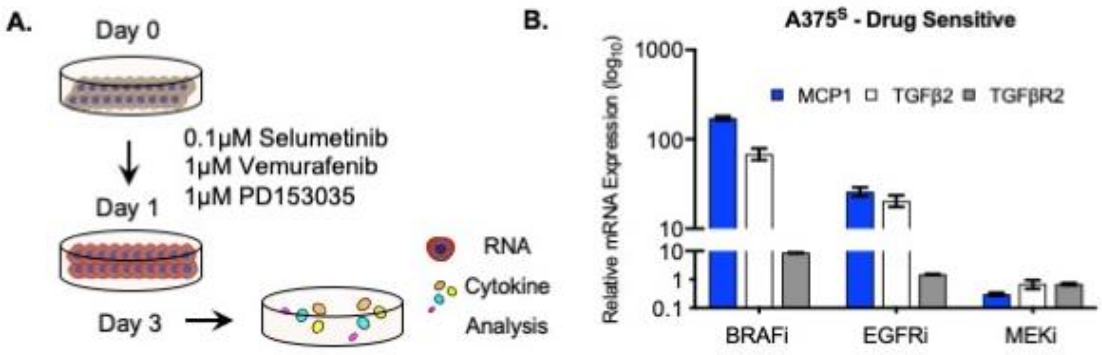

Figure 12: Drug stress induces the activation of TGFβ and inflammation pathways

(A) Schematic of drug stress assay and downstream analysis **(B)** Q-PCR of targeted genes show that EGFR (1μM PD153035), BRAF (1μM vemurafenib) and MEK (0.1μM selumetinib) drives a therapeutic stress response in A375 and increase in TGFβ related genes and pro-inflammatory MCP1. Data are presented as mean ± s.e.m.
(B)

The presence of vemurafenib, PD153035 and selumetinib in static cultures does not drive αSMA$^+$ activation in normal fibroblasts or CAF cell lines despite cell line variations in αSMA$^+$ at steady state (**Figure 13**). Components of the bioactive drug-stressed secretome from A375 conditioned media (CM) induces subsets of the αSMA$^+$ CAFs and normal fibroblasts (TP1170), (**Figure 14**). Interestingly, despite originating from the same patient and localized tumor site, 50236 distant CAFs exhibit more αSMA$^+$ induction in comparison to the 50236 adjacent CAFs. (**Figure 13-14**). Thus, we examined how CAFs responded to melanomas with and without drug stress.

The indirect coculture strategy is depicted in schematic form. Briefly, fibroblasts established from human samples and primary melanomas were seeded in transwell coculture with melanomas in the presence of a therapeutic stressor. Where indicated, fibroblasts were pretreated

with clinically relevant TGFβR inhibitor vactosertib or SB431542, and fibroblast growth factor (FGF) receptor BCJ398. After washing, therapy sensitive melanomas A375 or metastatic WM1158 cells were seeded and EGFR, BRAF, or MEK with-or-without TGFβR/FGFR inhibitors were added after 24 hours ('Day 2') and cocultures were allowed to interact for 72hr (**Figure 15A**). This system utilizes 0.4μm filters that do not permit transwell invasion or cell-cell contacts.

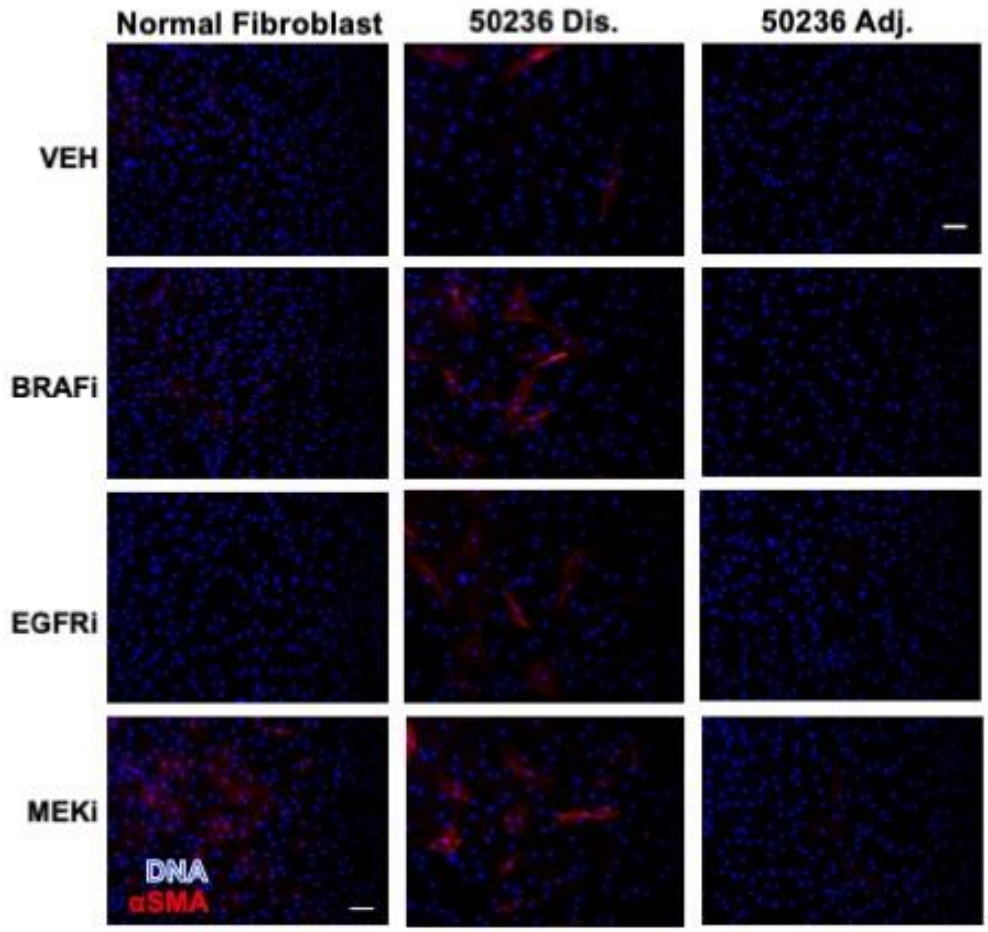

Figure 13: Fibroblasts tolerate small molecule inhibitors

Fibroblasts and CAFs show little induction of αSMA^{+} pathways in response to small molecules targeted to melanoma (n = 3 biological replicates). A representative image is shown for each condition at 72hrs. Vehicle low serum (2%FBS) and 1μM vemurafenib, PD153035 and selumetinib in low serum media. Original magnification 10x. Scale bar – 100μm.

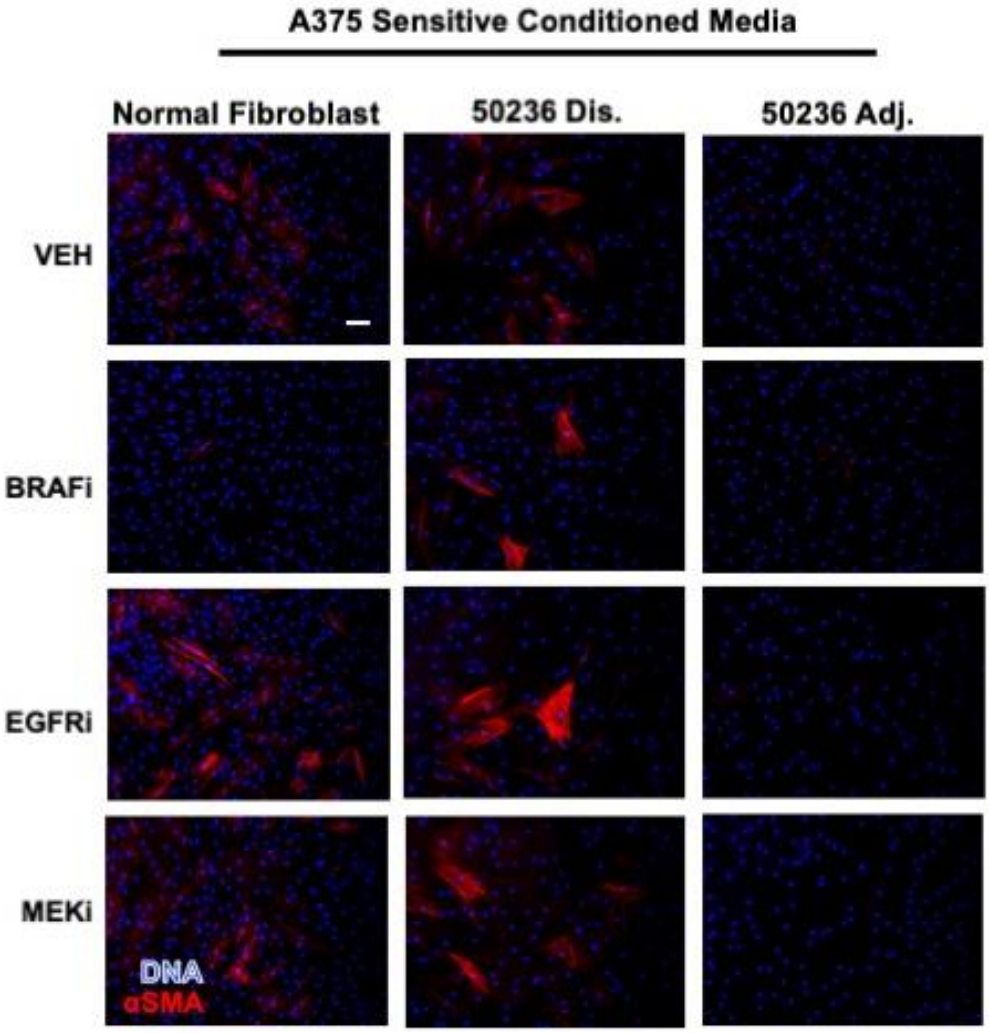

Figure 14: The drug-stressed secretome drives fibroblast activation

Fibroblasts and CAFs react to drug-stressed conditioned media (CM) in static monoculture. Induction of αSMA^{+} is observed in response to melanoma CM with vehicle while small molecules targeted to melanoma elicit obvious CAF and fibroblast responses. CM conditions: Vehicle low serum (2%FBS) and 1μM vemurafenib, PD153035 and selumetinib in low serum media (n=3 biological replicates). A representative image is shown for each condition at 72hrs. Original magnification 10x. Scale bar – 100μm.

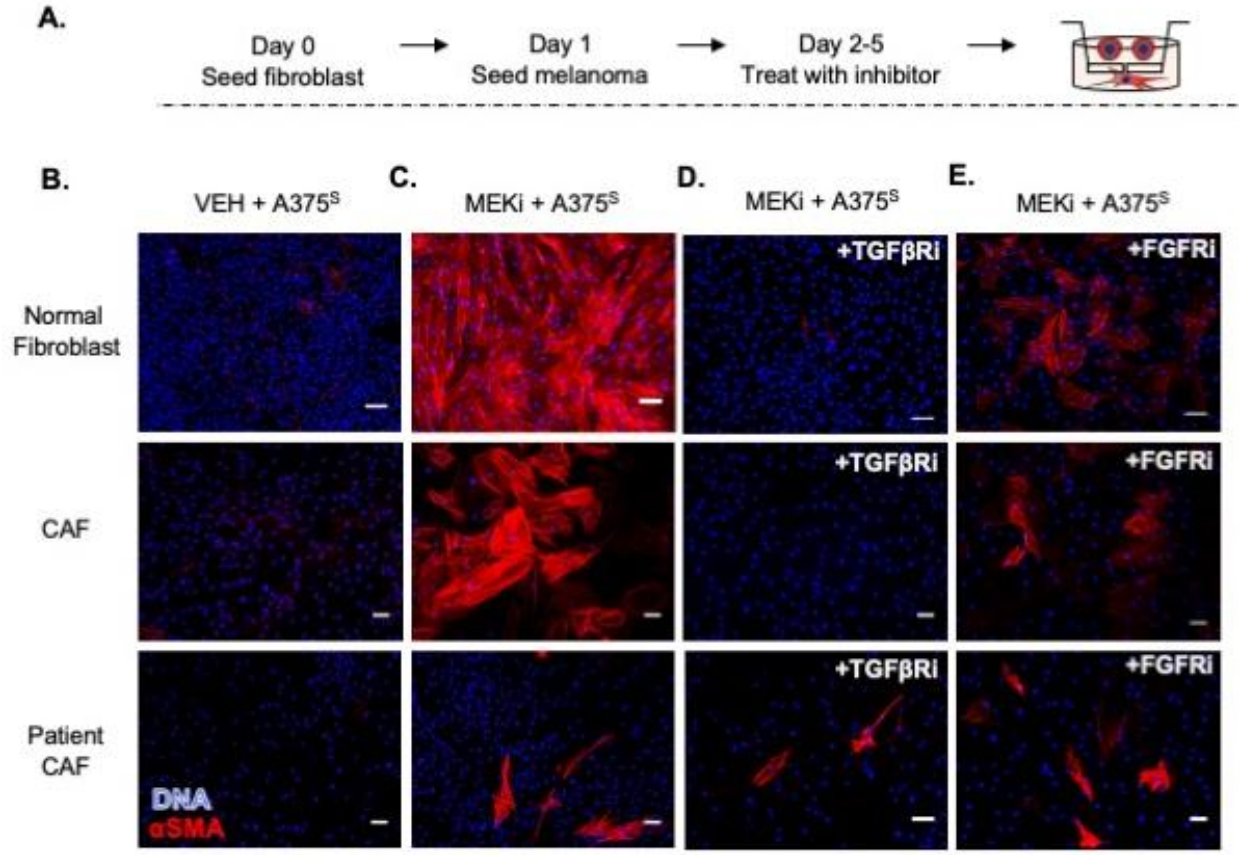

Figure 15: Therapy inflammation leads to fibroblast activation via the TGFβ pathway

(A) Schematic of drug stress cocultures and timeline to end point analysis after 72hrs. **(B)** Melanoma in coculture with normal, tumor associated, and patient derived CAFs does not induce αSMA. **(C)** Inhibition of MEK(i) in melanoma drives stress secretome and αSMA$^+$ fibroblast and CAF inflammation. **(D)** Pre-treatment to block TGFβR1/ALK5 (10μM SB431542) prior to MEK inhibition prevents TGFβ activation of αSMA in fibroblasts © Blocking FGFR with (1μM BCJ398) partially activates αSMA$^+$ fibroblast and CAF inflammation. A representative image is shown for each condition at 72hrs (n=3 biological replicates per condition, TP004 n=2 biological replicates). Original magnification 10x. Scale bar – 100μm.

Melanomas do not appear to activate αSMA$^+$ CAFs when there is an absence of therapeutic stress (**Figure 15B)**, however the addition of MEK inhibitor stimulates drug stress and leads to the activation of αSMA$^+$ in normal, CAF and primary patient derived fibroblasts (**Figure 15C**). The response in terms of αSMA$^+$ induction is heterogenous and varies by tissue ontogeny. There are

also discrepancies in terms of fibroblast proliferation evidenced by the quantity of cell nuclei per field, despite controlling for initial seeding density.

Given that TGFβ is transcriptionally induced during drug-stress and released from melanoma during invasion, we next examined if TGFβRI/ALK5 inhibitor SB431542 could prevent αSMA^+ CAF induction in MEK stressed melanoma coculture. Overnight pretreatment with 10μM SB431542 effectively blocked fibroblast and CAF αSMA expression (**Figure 15D**). The most dramatic response in normal neonatal fibroblasts is contrasted by fixed responses in CAFs which are phenotypically distinct and less plastic in response to TGFβ blockade.

Interestingly, Bordignon et al., report that TGFβ inhibition in fibroblasts results in activation of the fibroblast growth factor (FGF) signaling axis. The authors observed activation of TGFβ signaling when treated with FGF inhibitor BGJ398, resulting in αSMA expression that was associated with squamous cell carcinoma EMT and invasion. Conversely, TGFβ inhibition led to FGF activation that is more closely with CAF stimulation of macrophage inflammation (**Bordignon et al., 2019**). This report led us to examine the effect of FGF inhibition in our transwell coculture system where we observed that blocking FGF stimulates CAFs, even in the absence of drug stress. The addition MEKi to BGJ398 treated fibroblasts serves to further the activation and expression of αSMA regardless of the stromal cell type (**Figure 15E, Figure 16 – right**).

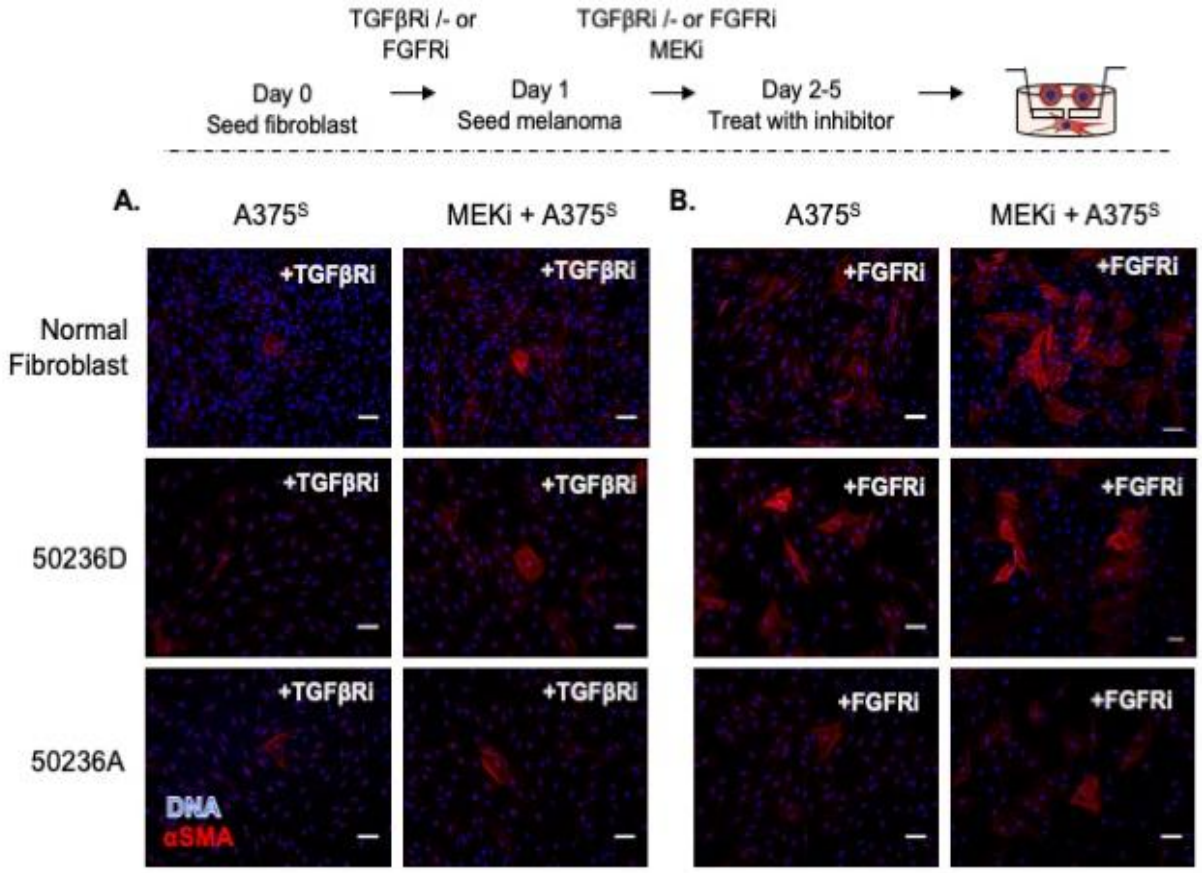

Figure 16: Clinical inhibitor of TGFβ blunts while FGFR activates CAFs

(A) Pretreatment of CAFs with clinically relevant TGFβ inhibitor vactosertib (1μM, TEW-7197) does not drive αSMA activity and suppresses CAF transformation during MEKi induced melanoma stress ('MEKi + A375$^{S'}$). **(B)** Conversely, inhibition of FGFR drives atypical αSMA activation in static-coculture and augments CAF transformation in drug-stressed cocultures. A representative image is shown for each condition at 72hrs (n=3 technical replicates). Original magnification 10x. Scale bar –

3.2.3 Drug-stressed release of TGFβ supersedes immune inflammation in CAF activation

Coupling our findings to Obenauf et al., regarding the release of CCL2 in addition to TGFβ during drug-stress, we hypothesized that immune cells which are also depots for TGFβ, may drive

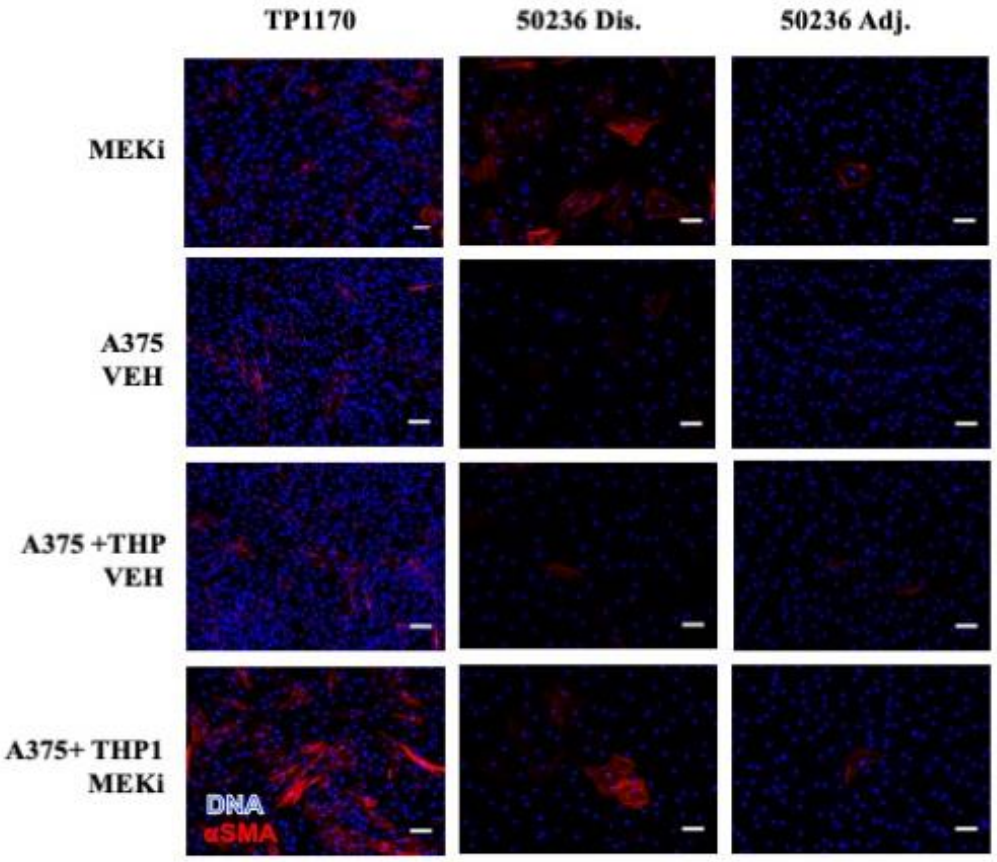

Figure 17: Immune inflammation does not appear vital for *in vitro* CAF transformation

(Row 1): The presence of MEKi (1μM selumetinib) does not disturb the quiescent stromal environment in CAFs and normal fibroblasts. **(Row 2)** The addition of uninflamed melanoma to coculture in the absence of drug (**Row 3**) and immune component does not drive CAF activation. **(Row 4)** Addition of MEKi induces pro-inflammatory secretome of melanoma leading to αSMA activation regardless of an immune component. A representative image is shown for each condition at 72hrs (n=3 technical replicates, n=2 melanoma cell lines). Original magnification 10x. Scale bar – 100μm

inflammation and *in vitro* CAF activation. To model these interactions, $1.5x10^4$ human peripheral blood monocytic cells (THP-1) were added to the both the upper and lower transwell

compartments, representing ~15% of the total cell population. After plating immune cells on the same day as melanomas, cocultures proceeded as indicated (**Figure 15A**). Having prior established that small molecules and uninflamed melanomas do not appear to activate αSMA in CAFs (**Figure 17, Row 1-2**), the addition of immune cells to static cocultures and the lack of αSMA induction in CAFs was anticipated (**Figure 17, Row 3).** However, the innate immune component was expected to contribute to CAF activation given the data showing CCL2 spikes in drug-stressed melanoma. Nevertheless, there was no apparent increase in inflammation and αSMA^+ CAFs in A375 and THP1 coculture during MEKi. In fact, the level of CAF activation appears comparable, if not less than what was observed in MEK inhibited A375 with melanoma alone (**Figure 17, Row 4 & Figure 15C**).

Given that immune cells do not appear necessary to drive αSMA^+ CAF expression, total cell actin was stained to assess actin stress fibers which are a hallmark of myofibroblasts. Interestingly, there appears to be an evolution in fibroblast actin stress fiber expression (**Figure 18**). Fibroblasts and CAFs are positive for actin stress fibers in the presence of MEKi (1μM selumetinib) but remain low in expression of CAF marker αSMA (**Figure 17-18, Row 1**). In the presence of uninflamed melanomas, a burst in proliferation is evidenced by the compaction of stress fibers as well as the morphological emergence of cell alignment in normal fibroblasts and CAFs (**Figure 18, Row 2**). The addition of naïve innate immune cells which do not trigger αSMA had limited impact on actin stress fibers (**Figure 18, Row 3).** However, melanoma and immune drug-stressed cocultures do lead to αSMA^+ CAFs (**Figure 17, Row 4**). These same cells also appear elongated and compacted with fibrillar organization of actin stress fibers; a common feature in fibrotic tissues (**Figure 18, Row 4**). Nearly all fibroblasts and CAFs are positive for actin stress fibers whereas subpopulations of CAFs are double positive for αSMA. Alternative activation of

CAF has also been observed during *in vitro* TGFβR and FGFR inhibition of patient fibroblasts independent of the genetic differences between individuals (**Storey JD et al., 2007**).

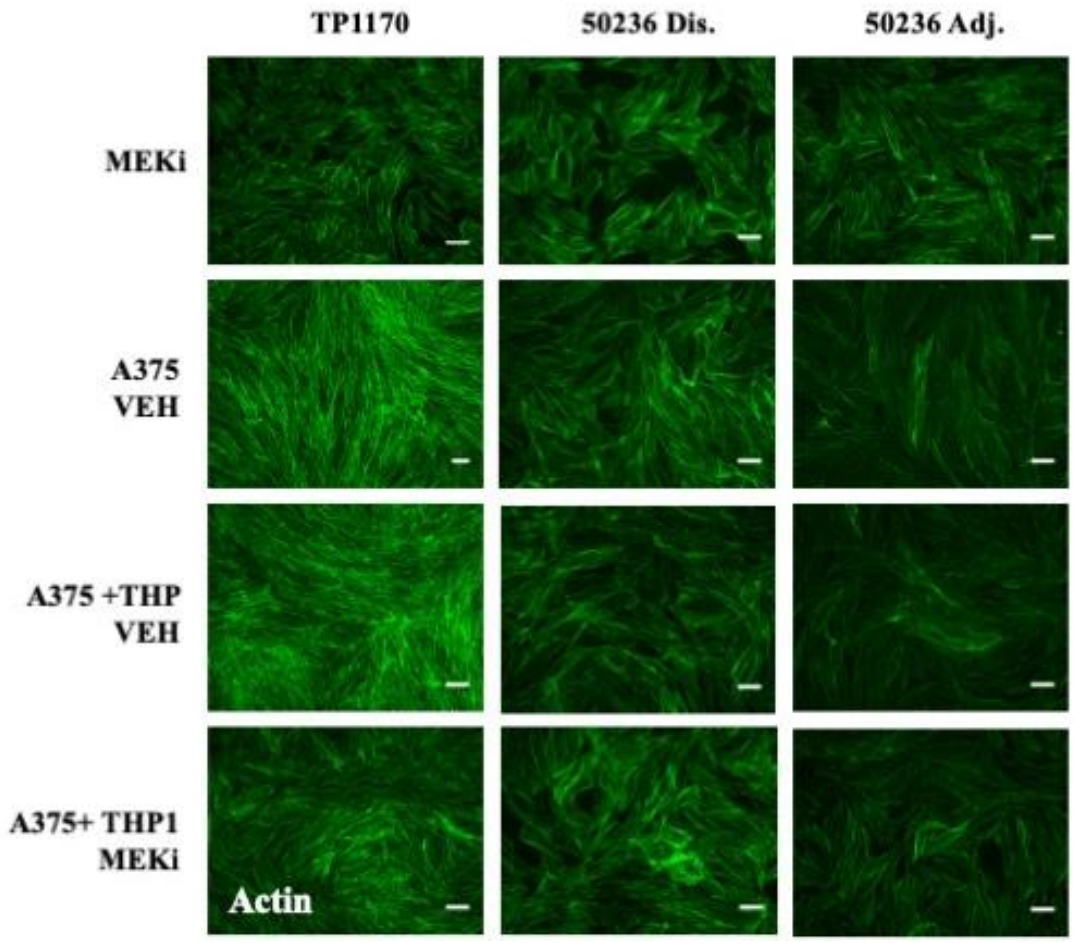

Figure 18: Actin stress fibers are activated but do not correlate with αSMA CAF

(**Row 1**) Fibroblast and CAFs have actin stress fibers in static coculture with MEKi (1μM selumetinib). (**Row 2**) Stress fiber compaction is evident with phenotypic and morphological responses to bidirectional signaling in coculture. (**Row 3**) Innate immune component does result in increased inflammation or stress fiber formation. (**Row 4**) Evidence of remodeling emerges with αSMA CAFs (Figure 17, Row 4) with stress fibers and organization of fibrillar arrangements. A representative image is shown for each condition at 72hrs (n=3 technical replicates, n=2 melanoma cell lines). Original magnification 10x.

CAFs are reported to exist in mutually exclusive states wherein TGFβ controlled gene expression drives αSMA, hallmarked by immune suppression and fibrotic ECM synthesis, while FGFR states oppose TGFβ to promote immune inflammation (**Bordignon et al., 2019**). This finding implies CAFs can coexist in heterogenous populations with αSMA^{+} TGFβ responsive cells driving differential responses to growth factors and cytokines in nearby negative $\alpha SMA^{(neg)}$ cells. However, $\alpha SMA^{(neg)}$ fibroblasts appear responsive to the pleiotropic signals in cell culture which manifests via the activation of stress fibers and proliferation. Still, inhibition of TGFβ in drug-stressed cocultures most directly impacts the expression of αSMA in fibroblasts.

In light of αSMA and actin stress fiber expression patterns observed of *in vitro,* which was also confirmed with metastatic melanoma WM1158 (data not shown), the necessary role of drug induced TGFβ expression and downstream role in activating αSMA appears evident. To confirm that TGFβ release is the result of drug-stress, the RNA-seq dataset for the therapy induced secretome by Obenauf et al., was examined for vemurafenib resistant A375© melanoma from *in vivo* tumors. The hypothesis driving this query was that drug-resistant melanomas lacking an inflammatory secretome would fail to activate αSMA CAFs due to the absence of TGFβ.

The induction of TGFβ2 and TGFRβII in therapy sensitive A375S tumors correlated with the *in vitro* A375S secretome (**Figure 19A, 11A**). However, vemurafenib induced expression of genes correlating to growth factors, the TGFβ pathway, and EMT/-drug resistance in A375S cells is virtually absent in vemurafenib resistant melanoma (A375R) (**Figure 19A**). Interestingly, there is a detectable 1.26-fold increase of EGFR transcript in A375R which correlates to an observed increase in protein expression for PD15035 (P) and vemurafenib (V) resistant A375R relative to the sensitive parental line (S) (**Figure 19B, left**). A similar trend in EGFR expression is observed in vemurafenib resistant W983B and in PD153035 resistant WM1158 (**Figure 19B, middle &**

right). In most cases where EGFR is upregulated, phospho-ERK trends while phospho-AKT induction is transient. From the expression dataset, it appears that downregulation in TGFβ transcription is associated with an uptick in EGFR.

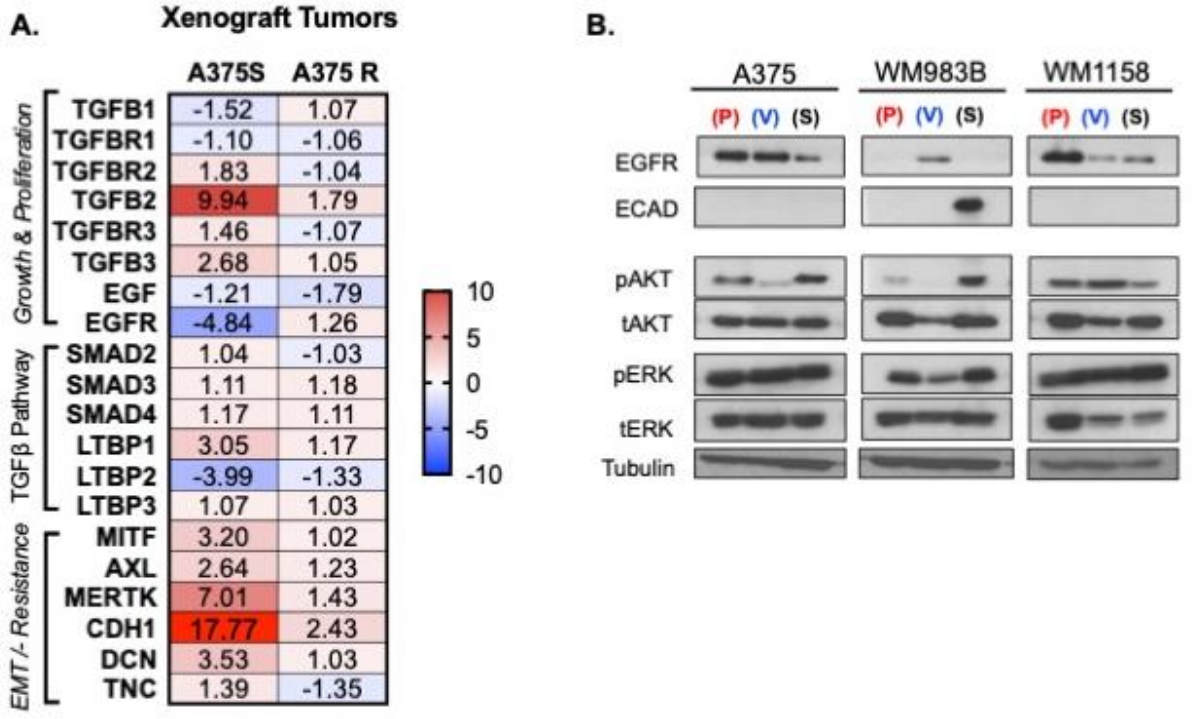

Figure 19: Drug-resistance blunts the *in vivo* therapy induced secretome

(A) Heat map showing log fold change of gene induction between sensitive A375S and resistance A375R treated xenograft tumors Obenauf *et al.* 2015 (GEO: GSE64671). **(B)** Protein analysis of PD153035 (P), vemurafenib (V) and parental (P) melanoma.

The limited induction of the TGFβ pathway of A375R tumors trends with the suppression of genes linked to the EMT pathway. There is a reduction in MITF and AXL which Tirosh et al., linked to drug resistance, in addition to epithelial marker E-cadherin and anti-invasive DCN and pro-invasive TNC (**Figure 19A, 'A375R'**). Thus, the pathways that feed forward in establishing

proliferative ability, suppression of cell cycle control, EMT/-drug resistance, and TGFβ appear to turn off in the absence of vemurafenib stress.

To test how therapy-resistant melanomas impact CAFs, melanomas were cultured in the presence of small-molecule inhibitors for ~8weeks. Clones were selected based on phenotype and response, expanded and analyzed for protein expression. (**Figure 19B**). Resistant clones displaying stable increases in growth factor expression and constitutive activation of ERK and AKT were used to determine if drug-resistant melanomas can inflame CAFs.

Cocultures with vemurafenib A375R melanomas show little activation of αSMA in normal and cancer-associated fibroblasts in the absence of drug-stress (**Figure 20, Row 1**). The addition of vemurafenib (BRAFi) to resistant cells also fails to drive the release of the drug-stress secretome. However, inhibition of BRAF in melanoma paradoxically elicited a weaker induction of αSMA in fibroblasts in comparison to selumetinib and PD153035 at the same and is concentration (**Figure 21**). This finding prompted the use of selumetinib throughout this study.

The use of MEK inhibitors is also of particular relevance for vemurafenib resistant melanoma, especially given that combined BRAF and PI(3)K/AKT/mTOR is reported to control the Given that the reactive secretome is activated in melanoma in both a drug-specific and dose-dependent situation – this secretome varies slightly between *in vitro* and *in vivo* conditions. We elucidated from the therapy-stressed secretome that the TGFβ pathway is consistently induced during drug-stress. In cocultures, this TGFβ containing secretome consistently activates αSMA^{+} pathways in fibroblasts driving conversion to CAFs (confirmed by western analysis – **Figure 22**). This activation proceeds predominantly through TGFβ, which when blocked using small molecules SB431542 and vactosertib, blunts αSMA^{+} CAFs and pSMAD (**Figure 22**).

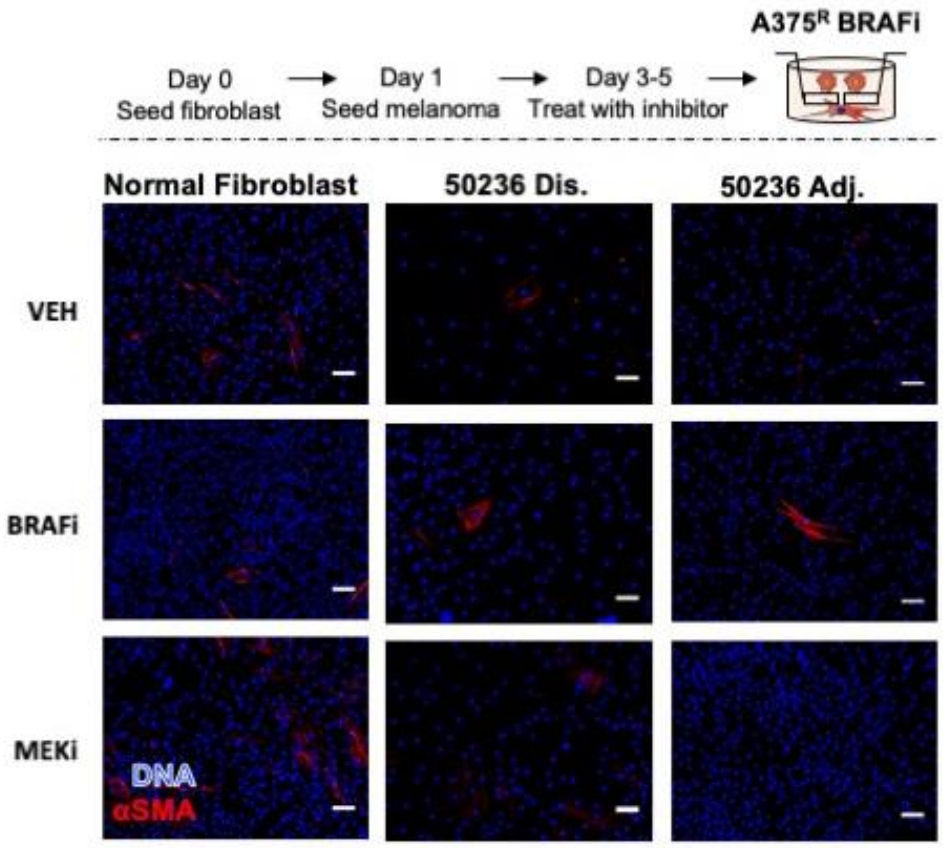

Figure 20: Drug-resistant cells lack an inflammatory secretome

Fibroblasts and CAFs show little induction of αSMA^{+} pathways in response to small molecules targeted to vemurafenib resistant melanoma (n = 3 technical replicates). A representative image is shown for each condition at 72hrs. Vehicle low serum (2%FBS) and 1μM vemurafenib and selumetinib in low serum media. Original magnification 10x.

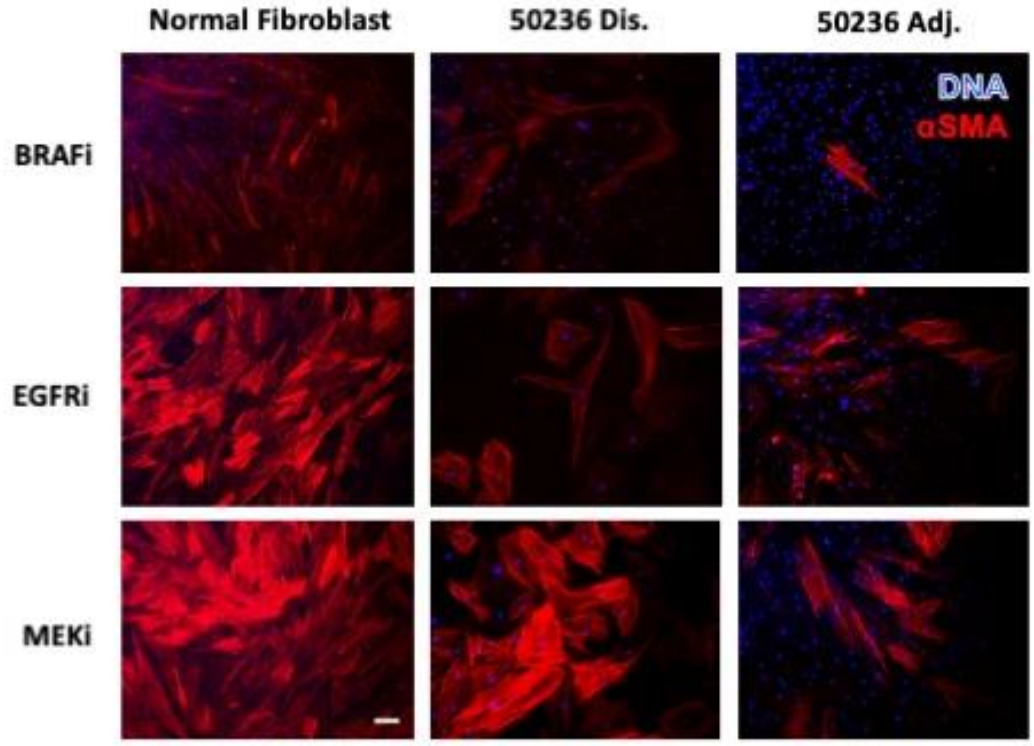

Figure 21: Targeted inhibitors drive differential *in vitro* coculture CAF responses

(**Row 1**) Inhibition of BRAF using 1μM vemurafenib drives αSMA expression while (**Row 2**) 1μM PD153035 and (**Row 3**) selumetinib induce marked increases in αSMA expression in normal fibroblasts and CAFs in coculture with A. A representative image is shown for each condition. (n=3 technical replicates). Original magnification 10x. Scale bar – 100μm.

Alternative TGFβ activation was observed in FGFR suppressed fibroblasts, which is linked to TGFβ and FGF dualism in regulating CAFs between pro-fibrotic and immune inflammatory phenotypes. This was exemplified here by CAFs maintaining actin stress fibers while subpopulations of CAFs were observed to be double positive for αSMA^{+} and stress fibers. Furthermore, CAF responses are not uniform in terms of SMAD activation and αSMA induction in response to TGFβ highlighting the challenges associated with targeting these heterogenous

populations. However, human data and animal studies (**Figures 4-9**) show that αSMA induction occurs simultaneously in micrometastatic melanoma outgrowth and inflammatory ECM remodeling. Therefore, we sought to determine how CAFs drive melanoma outgrowth *in vitro* using dense-tissue mimics referred to as spheroids.

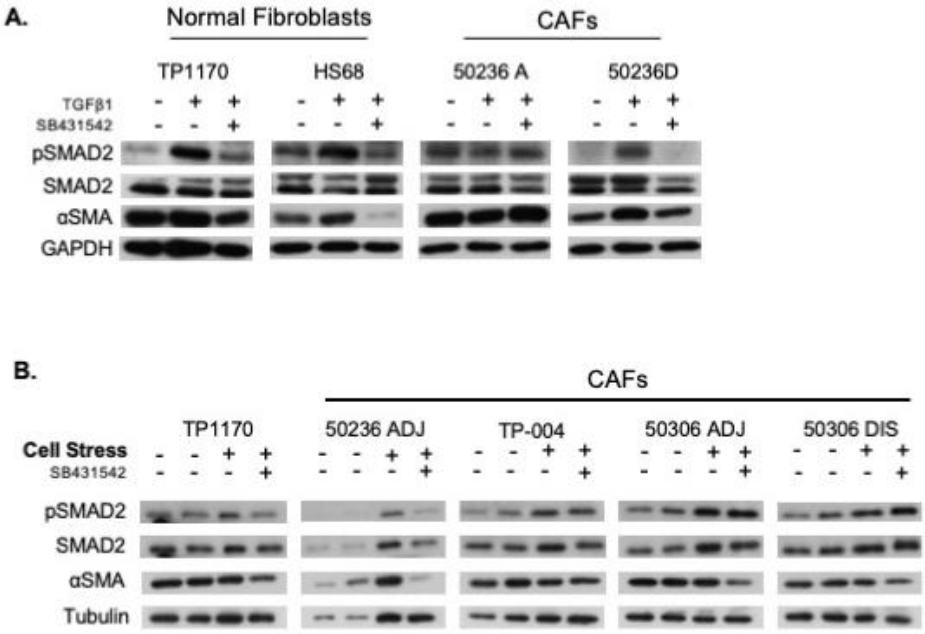

Figure 22: Western analysis of TGFβ and cell stress induction of αSMA in CAFs

(A) TGFβ induces intracellular SMAD2 to activate the pathway and expression of αSMA in normal and cancer-associated fibroblasts while pre-treatment to block TGFβR1/ALK5 (10μM SB431542) after 24hrs abolishes pSMAD2 and αSMA. **(B)** MEK inhibition of A375 in coculture with fibroblast and CAFs drives activation of αSMA which is suppressed by varying degrees with TGFβR1/ALK5 (10μM SB431542) after 24hrs. (n=2 technical replicates per condition).

3.2.4 Cancer-associated fibroblasts drive in vitro melanoma outgrowth

There are multiple studies linking the presence of CAFs to the progression of primary tumors, including melanoma (Kaur et al., 2015). To ascertain how CAFs impact melanoma outgrowth in combination with the drug-stressed secretome, a modified transwell coculture system

was utilized. Briefly, to examine the effect of inflamed fibroblasts on melanoma invasion, a spheroid coculture system wherein fibroblasts and CAFs are seeded directly on to the transwell membrane followed by the deposition of collagen into which a spheroid is embedded, was designed (**Figure 23A**).

After embedding, spheroids were allowed to interact with the soluble secretome, both with and without drug-stress. Invasive outgrowth was measured after 72 hours. Metastatic WM1158 spheroids outgrow ~4-fold in comparison to the Day 0 area in the absence of fibroblasts or CAFs (**Figure 23B**). That addition of LPS/EGF to stimulate EGF mediated motility in these $EGFR^{HI}$ expressing cells resulted in 5.3-fold increase and 37% more outgrowth compared to no stimulation/- no fibroblast control. However, this was not statistically significant.

Cocultures with normal fibroblast (TP1170) suppressed outgrowth (3.25-fold, Day 3) relative to the unstimulated and stimulated controls (**Figure 23B, 'nrml fib'**). This is consistent with reports that co-implantation with healthy fibroblasts results in tumor stasis (**Kullari R., 2016**). Young CAF, 50306 tumor distant and adjacent drive 4.9 and 5.3-fold increase in invasion, respectively. This effect is comparable to the LPS/EGF inflammatory stimulus control. Lastly, aged CAFs, 50236 drive a 5.13 and 6.33-fold increase in invasion between tumor distant and adjacent, respectively. Representative images of spheroid invasion at Day 3 show how CAFs augment melanoma invasion (**Figure 23C**). A related study by Kaur et al., reported that melanoma

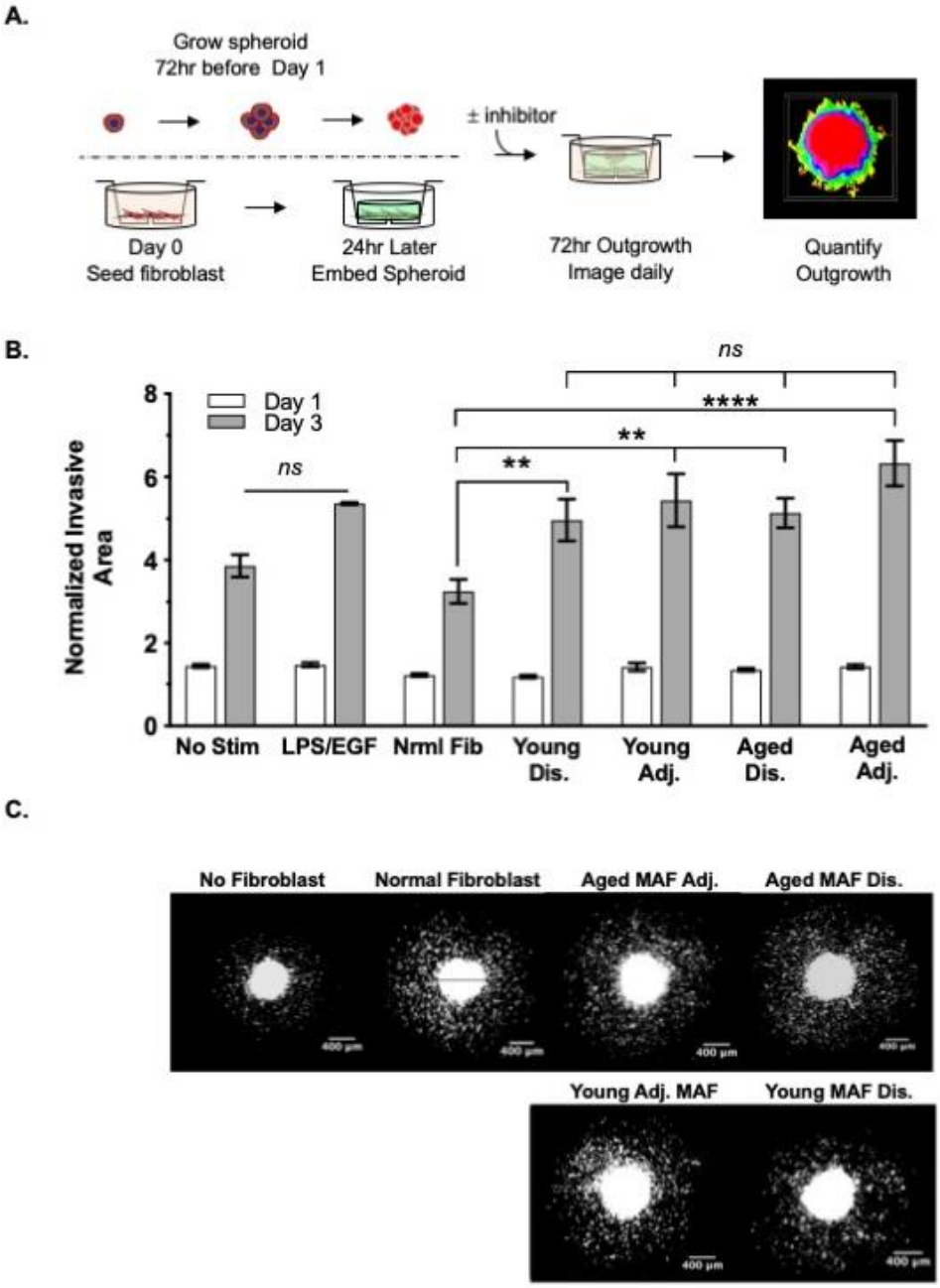

Figure 23: Cancer associated firboblasts augment the invasion of metastatic melanoma

(A) Schematic timeline of co-culture invasion assay. **(B)** Invasion of melanoma spheroids in coculture with normal and cancer associated fibroblast cell lines derived from an aged patient. Invasion was quantified by ImageJ (n=2 fibroblasts per group, n ≥ 3 spheroids per group; Day 3, ANOVA ± s.e.m., $P < 0.01$ ***, 0.0001 ****; Bartlett's statistic corrected, $R^2 = 0.85$ – original magnification 4x). Adj, tumor adjacent; Dis, tumor distant. **(C)** Representative images of 72hr outgrowth for selected conditions. Scale bar – 400μm.

CAFs, which are disproportionately common in older individuals, secrete a proto-oncogene-INT 1 (WNT1) antagonist secreted frizzled related protein 2 (sFRP2). This molecule drives signaling cascades that alters β-catenin thereby driving pro-epithelial MITF down to promote invasion. This bi-directional signaling loop is purported to increase in aging and ultimately drives metastasis of melanoma (**Kaur et al., 2015**). Although the focus here is not on aging, and instead on inflammation driven remodeling of the tumor-ECM, synergy or plasticity between these pathways during progression cannot be ruled out. Our results demonstrate that normal fibroblasts suppress invasion while CAFs promote melanoma outgrowth. The tumor-stroma coculture spheroid model supports our initial hypothesis that bi-directional signals in the TME converge to drive pro-tumorigenic programs. Based on our previous findings demonstrating that small molecules drive stress in melanoma and inflame the stroma, addition of MEK inhibitor to cocultures was tested to determine if CAFs promote melanoma invasion.

The growth of A375 spheroids, which form a loose spheroid, show moderate outgrowth in culture with normal fibroblasts and CAFs on Day 3 (**Figure 24A, *i***). Addition of 1μM MEK1/2 inhibitor selumetinib suppressed Day 3 invasive outgrowth relative to untreated control and resulted in smaller spheroids, ($p<0.05$) (**Figure 24A, *ii***). Conversely, highly metastatic WM1158 which are rounded in 2D and amoeboid during invasion, readily invade collagen (**Figure 24B, *i***). The same MEK suppressive effect on invasion also occurred in WM1158 spheroids, which were observably smaller, though this was *not significant* (*n.s.*) with respect to the untreated control (**Figure 24B, *ii***). WM1158 spheroid cocultures with normal fibroblasts also show comparable outgrowth to Day 3 untreated invasive outgrowth from the assays in (**Figure 23B, *i***). Therefore, the reduction of invasion and smaller spheroids observed in this assay can be reliably attributed to MEK inhibition.

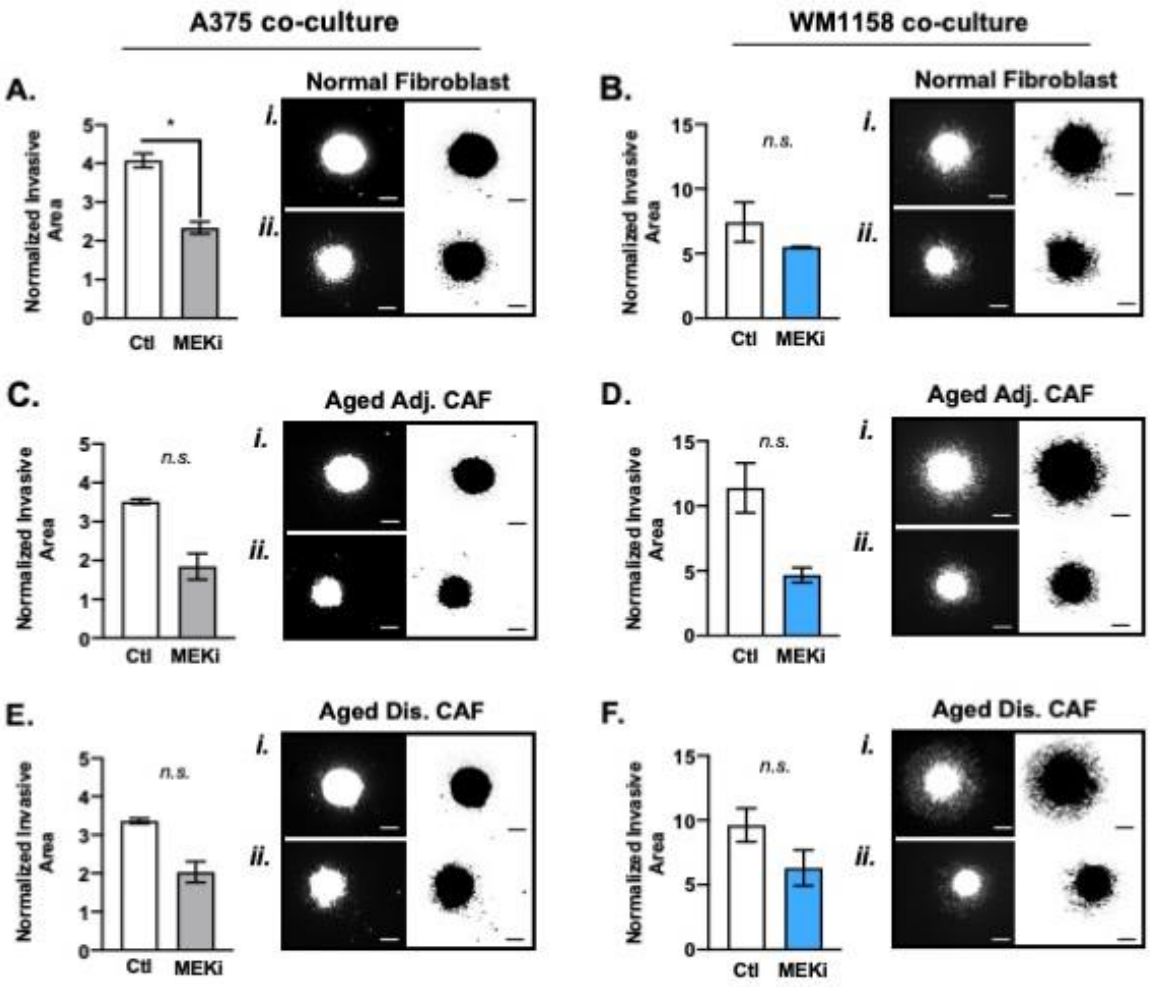

Figure 24: Inhibition of MEK in melanoma limits outgrowth but not invasion

(A) Invasion of malignant A375 and **(B)** metastatic WM1158 spheroids in co-culture with normal fibroblast (TP1170). (**C**) A375 and **(D)** WM1158 spheroids cancer associated fibroblast 50236 Adj. and A375 and **(F)** spheroids with 50236 Dis. (cell lines derived from an aged patient, 50236). Invasion was quantified by ImageJ (n=2 fibroblasts per group, n ≥ 3 spheroids per group; Day 3, Student's unpaired t-test ± s.d. *P*< 0.001 ***, 0.0001 ****; Day 3 Welch's correction, original magnification 4x). Representative images of 72hr outgrowth for selected conditions. Adj, tumor adjacent; Dis, tumor distant, **©** = *control*, ***ii*** **=** *MEKi*. Scale bar – 200μm.

Aged tumor adjacent CAF, 50236 Adj., had limited impact on untreated malignant A375 outgrowth while MEKi prevented outgrowth of the spheroid (**Figure 24C, *i-ii***). In contrast, cocultures of 50236 Adj. and WM1158 spheroids resulted in greater than 2-fold increase in

invasive area compared to MEKi treated spheroids (**Figure 24D, *i-ii***). The same trends were consistent for both A375 and WM1158 spheroid cocultures with 50236 Dis., (**Figure 24E-F, *i-ii***). Thus, inhibition of MEK limited spheroid outgrowth for both A375 and WM1158 spheroids in all conditions, despite the pro-inflammatory secretome and CAF activation. However, MEK inhibition of melanomas does not prevent invasion. Instead, MEKi appears to activate a subset of invasive cells while restricting growth of the primary mass. A375 spheroids shift from collective outgrowth in untreated conditions to invasive sentinel cells due to MEKi induced stress. For both cell types, the result of MEKi is smaller tumors with more invasive cells at the periphery. Therefore, MEKi induced stress in melanoma limits outgrowth, seemingly independent of CAF activation during the 72hr assay. Prompted by this finding and results from our *in vivo* studies, we hypothesized that ECM remodeling may precede the contributions of a bioactive and inflamed stroma. Immunohistochemical evidence here showing ECM-remodeling during the early phases of micrometastatic outgrowth suggested that ECM may have key roles in regulating invasion.

3.2.5 The inflamed stroma produces pathological matrix that promotes invasion

We have previously reported on the turnover of matrix that occurs during melanoma progression. During invasion the proportion of homeostatic proteins such as SLRPs and collagens are diminished while immature matrix is upregulated (**Hood BL et al., 2010**). These findings prompted experiments to build on our understanding of how inflamed stroma may modify expression of certain matrix proteins to promote melanoma invasion.

In order to probe the effects of matrix on melanoma invasion, we constructed collagen plugs to study the vertical migration of melanoma (by courtesy of Jelena Grahovac). Briefly, control collagen gels and collagen mixed with decorin (DCN) or TNC (tumor-ECM mimic) were

prepared to model the pathophysiological contributions of select ECM proteins that accompany fibroblast inflammation. Metastatic WM1158 melanoma were seeded on top of collagen (COL1), COL1-DNC, COL1-TNC, and COL1-DNC-TNC.

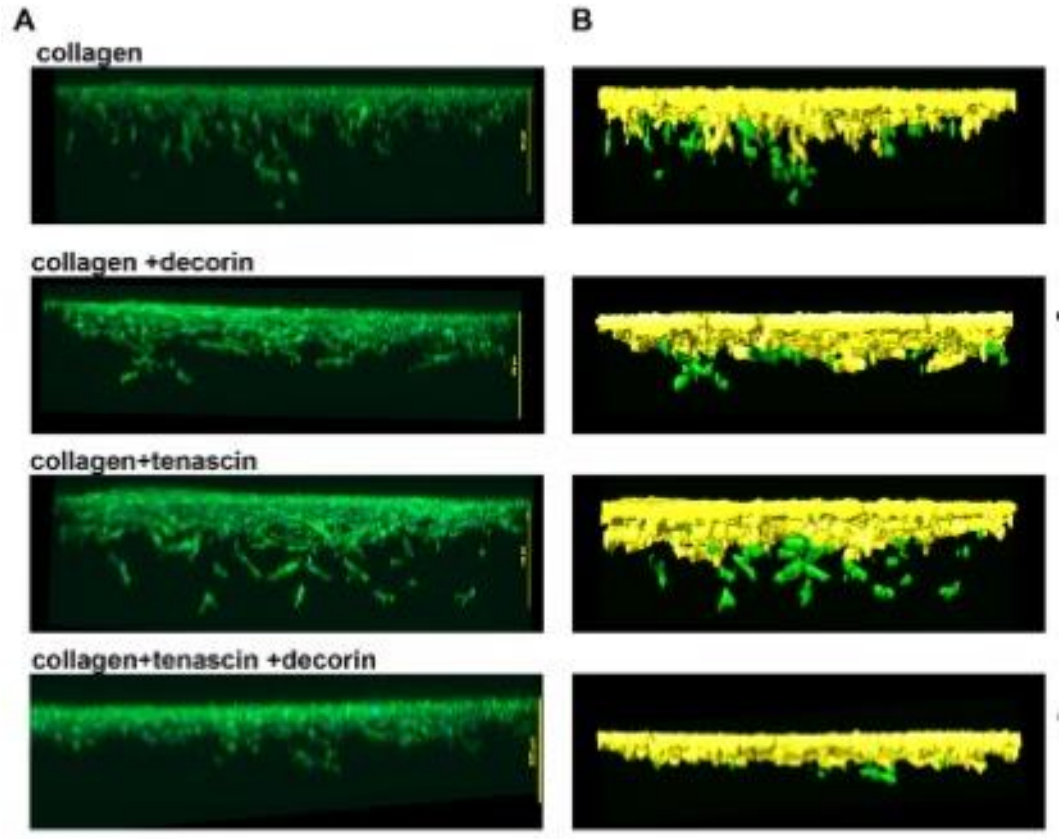

Figure 25: Normal ECM halts – while Tumor ECM – promotes invasion

(A) WM1158 invasion in collagen gels with representative ECM proteins to mimic normal and tumor ECM synthesized by CAFs. **(B)** Invasion analysis to detect cell volume and detachment.

WM1158 readily invaded into 3mg/ml COL1 gels via collective (yellow) and single cell invasion (green) (**Figure 25A-B, Row 1**). The addition of anti-invasive and tumor inhibiting DCN (4μg/ml) into collagen gels suppresses collective invasion compared to control COL1 (**Figure 25A-B, Row 2**). Conversely, the addition of tumor promoting TNC (4μg/ml) to collagen

significantly promotes WM1158 invasion (**Figure 25A-B, Row 3**). This agrees with our previous work wherein genetic transformation of radially invasive WM983A melanoma with EGFL-containing TNC was sufficient to drive vertical invasion with TNC localized to the invasive front (**Grahovac J et al., 2013**). Enrichment of TNC at the invasive front has been reported in micrometastatic breast nodules and reviewed by Lowy et al. (**Oskarsson T et al., 2011; Lowy CM et al., 2015**). Here, we observe enrichment of TNC *in vivo* within micrometastases, while COL1-TNC gels definitively shows that this anti-adhesive protein promotes single cell invasion; detached cells (green) and collective tumor mass (yellow) as quantified by Imaris software (**Figure 25B, Row 3**). Addition of DCN into pro-invasive COL-TNC gels dramatically suppressed WM1158 invasion (COL-DCN-TNC) (**Figure 25A, Row 4**). Decorin also limits single cell invasion (green) and collective invasion despite the presence of TNC (**Figure 25B, Row 4**).

To validate these results in a more representational melanoma microenvironment, we tested the invasion of WM1158 cells in three-dimensional organotypic skin organ cultures (SOCs). These SOCs develop forming a cornified epidermis, granular and spinous layer, over top of a developing basal keratinocyte layer which generates the basement membrane ©. Normal neonatal fibroblasts (TP1170) are embedded into 3mg/ml collagen gels to form the dermal compartment (D) (control SOC, Figure 26A). Seeded WM1158 cells invaded into the dermal compartment of SOCs within 20-day time frame, both collectively and as detached cell clusters (Figure 26B). The addition of DCN (4μg/ml) into the dermal compartment prevented melanoma cells from invading into the

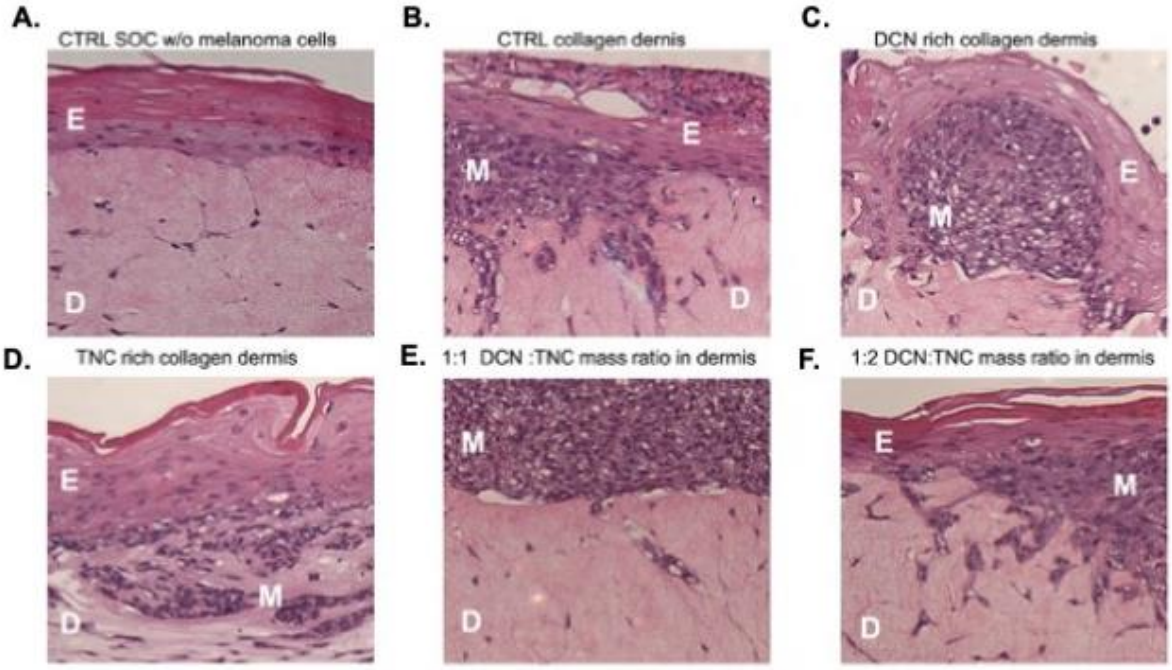

Figure 26: Tumor ECM drives progression while normal ECM blocks invasion

(A) Collagen control skin matures with development of an epidermis ©, basal keratinocytes, and dermis (D). **(B)** The addition of WM1158 melanoma (M) to collagen (CTRL) skins **(C)** Mature matrix protein DCN blocks WM1158 invasion. **(D)** Tumor-ECM matrix protein TNC in collagen drives marked invasion. © Mature matrix DCN blocks the tumor promoting effect of TNC. **(F)** Changing the tumor-ECM ratio 2:1 TNC to DCN re-establishes invasion into the tumor stroma. A representational image is shown for each condition.

dermis. The bulk of the tumor growth appears confined to the epidermis (**Figure 26C**). Conversely, the addition of TNC (4µg/ml) markedly induced melanoma invasion into dermis, with advancing collective invasion (**Figure 26D**). Based on the stochiometric ratios used here, tumor inhibiting or promoting ECM can be titrated to regulate invasion in a dose-dependent manner (1:1, DCN: TNC ratio 4µg/ml each (w/w)). Interestingly, WM1158 in COL1-DCN skins exhibited stalled invasion, but remained proliferative in the epidermis © (**Figure 26C**). Increasing the TNC:DCN ratio 2:1 results in WM1158 that respond to the pro-invasive effects of TNC (4µg/ml: 8µg/ml, (w/w)). Based on these findings, the role of ECM in acting as a barrier to invasion is apparent.

Experimentally remodeling the ECM with wound healing protein TNC to mimic the tumor ECM promotes rapid advancing melanoma. This can be halted via the incorporation of mature, anti-invasive, ECM molecule DCN which suppresses tumor progression. This is of interest given that CAFs in drug-stressed spheroid cocultures did not appear to blunt the cytotoxic effect of MEK inhibition or single cell invasion. However, the use of ECM molecules synthesized by normal fibroblasts such as DCN, or TNC by melanoma and CAFs exert an immediate effect on melanoma invasion and progression. Taken together, there is evidence to suggest that ECM remodeling and altered signaling likely converge to influence multiple facets of melanoma invasion and resistance.

3.3 Discussion

3.3.1 Drug resistance in melanoma

Common treatments such as chemotherapy, targeted therapies and even immunotherapy regimens confer modest survival benefits to patients with metastatic melanoma. Primary lesions clinically exhibit a robust response that is lost overtime and generally muted in combating metastasis. Briefly, vemurafenib targets the commonly mutated $BRAF^{V600E}$ mutation which is present in approximately 50% of melanomas (Cancer Genome Atlas Network, 2015). However, evolving tumors adapt to treatment and develop resistance that drives partial responses in most patients (**Sosman JA et al., 2012; Chapman PB et al., 2011; Flaherty KT et al., 2010**). Resistance is primarily achieved by mutation or convergent alternative activation of multiple signaling axes.

In melanoma, the mitogen activated protein kinase (MAPK) pathway proceeds via activation of rapidly accelerated fibrosarcoma serine/threonine kinase (RAF; ARAF, BRAF, c-RAF) which signal downstream to extracellular-regulated kinase (ERK) and phosphoinositide 3-kinase (PI(3)K-AKT-mTOR) or AKT. This cascade activates multiple tumor processes that drive initiation, progression and therapy resistance (**Lito P et al., 2013**). The development of FDA approved inhibitors vemurafenib, dabrafenib (BRAF), and trametinib (MEK) which are often used to shut this pathway down in melanoma, also lead to the alternative activation of non-genetic programs that are now gaining appreciation as drivers of resistance.

Therapeutically targeted melanomas are antagonized by drugs (*incl.* chemotherapy) resulting in external and internal stimuli that lead to the activation of cell stress. This stress triggers a broad range of cellular functions. Vemurafenib stress induces endoplasmic reticular stress and mediated apoptosis in melanoma while also driving activation of a therapy-induced secretome that promotes resistance and progression (**Beck D et al., 2013; Obenauf AC et al., 2015**). The therapy-induced stress secretome contains multiple inflammatory growth factors and cytokines that results in tumor self-seeding and recurrence (**Kim MY et al., 2009**). This prompted the investigation of how the drug-stressed secretome stimulates an inflammatory matrix and whether this promotes metastatic melanoma.

3.3.2 The complex tumor contexts of TGFβ

This thesis identifies the TGFβ-SMAD pathway as a driver of CAF activation and ECM remodeling in the TME. This process is outlined as a graphical abstract (**Figure 27**). Briefly, small molecule inhibitors were shown here to drive the inflammatory transcription of TGFβ, macrophage chemotactic protein 1 (MCP1/CCL2), in addition to the first report of a BRAF therapy-induced

secretome. This induced secretome contains intracellular SMADs, latent TGFβs, and genes related to EMT and ECM which are indicated in melanoma outgrowth. Here, TGFβ is implicated in the

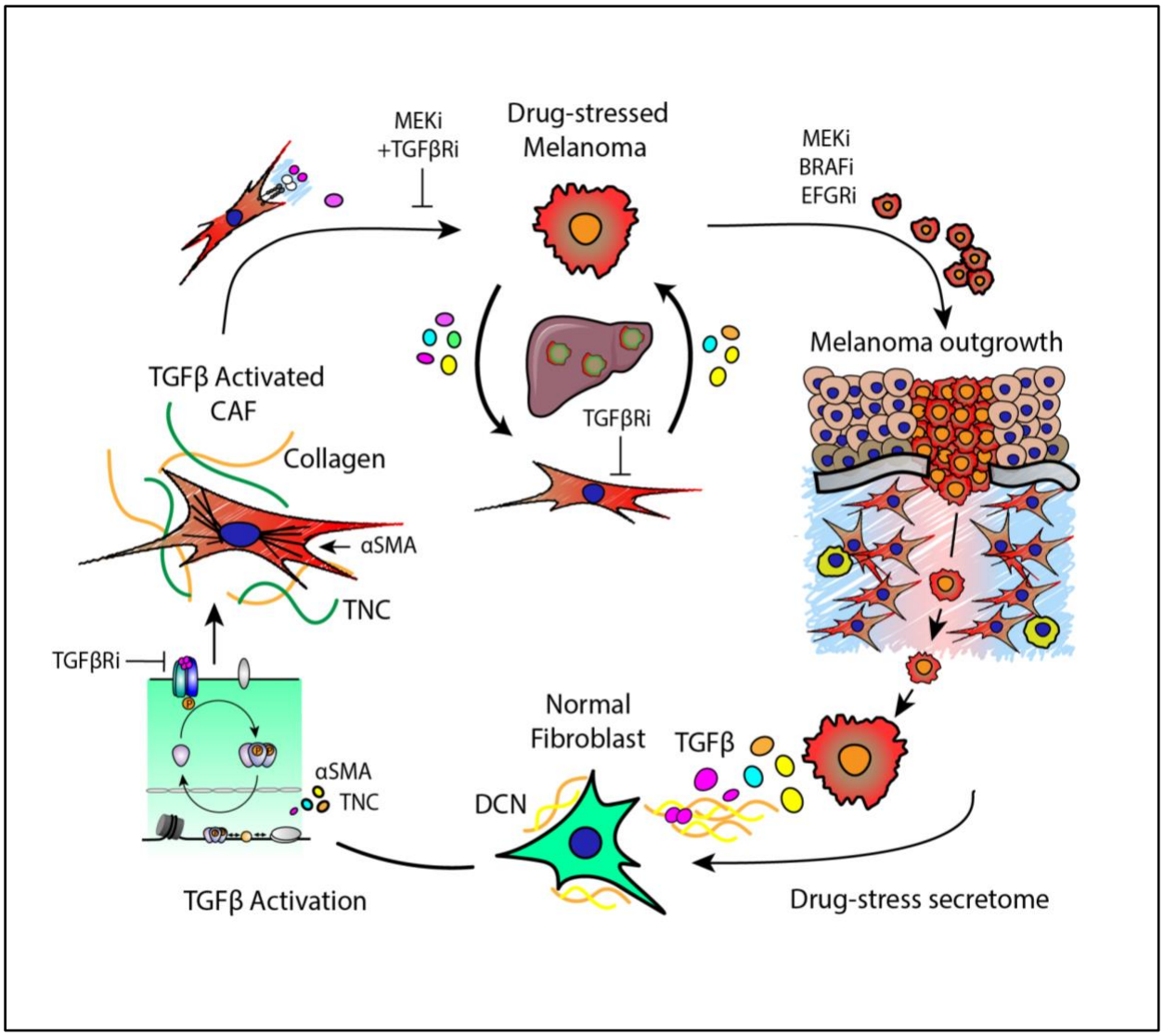

Figure 27: Graphical abstract of TGFβ driven inflammation and melanoma progression

Proposed TGFβ signaling loop involved in the activation of CAFs and proposed inhibitory strategies for treating metastatic melanoma.

pathological activation of cancer-associated fibroblasts (CAFs). This process, initiated by TGFβ binding to TGFβRI/II, activates intracellular phosphorylation of SMAD2/3 which forms heterotrimer complexes with SMAD4. These complexes transit the nucleus, driving genetic programs that impact the maintenance of CAFs, ECM synthesis and a CAF secretome. These pleiotropic signals manifest in the formation of complex and drug resistant TMEs.

Transforming growth factor βeta paradoxically has both tumor suppressive and supportive functions in cancer. The TGFβRI/II receptors are not directly coupled to pro-apoptotic pathways as we did not observe *in vitro* lethality in melanoma and CAF responses to TGFβ1. In fact, SMAD2/3 nuclear localization in A375 occurred 24-48hrs after treatment TGFβ1 and suggesting that TGFβ is not a key survival signal in melanoma (data not shown). Instead our data shows that TGFβ1elicits a rapid induction of p-SMAD in normal and CAF cell lines leading to reversible αSMA expression when blocked with TGFβRI/II inhibitors SB431542 or vactosertib. In cancer, cells are suspected to undergo selective pressure imposed by TGFβ exposure resulting in clones that can then undergo TGFβ-induced EMT (**David CJ et al., 2018**). Though the drivers of desensitized TGFβ responsive cancer cells are unclear, PI(3)K/AKT/mTOR (AKT) signaling stimulated by growth factors from the tumor stroma are emerging as candidates. Obenauf et al. reported that combined inhibition of RAF and AKT effectively disrupts outgrowth of drug resistant melanomas (**Obenauf AC et al., 2015**). However, the penetrance of these genetic treatments is limited owing to inflammation that spurs increasingly complex stromal networks.

Our results demonstrate that the stroma has varied impact and function in response to drug-stressed melanoma. Cocultures of melanoma and CAFs appears to elicit CAF activation primarily though soluble TGFβ. Future studies should probe whether specific SMADs trigger this function in CAFs using siRNA for transient studies. Stable knockdowns using the pLKO-shRNA vector

targeted to TGFβRI/II receptors in CAFs will deconvolute whether αSMA expression is exclusive to the TGFβ pathway. This is of particular importance as TGFβ has coreceptors in addition to the pleiotropic signaling capacities and modularity of this pathway.

Within the context of how TGFβ impacts stromal-mediated progression, findings from this thesis point to CAFs as the primary driver of metastatic melanoma. Our findings show a concomitant increase EGFR in higher and metastatic grade malignancy. Metastatic melanomas are also reported to secrete ~2-fold more TGFβ (pg/ml) *in vitro* compared to low-grade cell lines such as WM983A/B. In the context of melanoma, $TGF\beta^{HI}$ expression in localized invasion activates SMAD2/4 and leading to CITED1 transcription. CITED1 expression was associated with non-proteolytic amoeboid rounding. This TGFβ phenotype displays increased *in vitro* and *in vivo* invasion and poor outcomes in patient samples (**Cantelli G et al., 2015**). Our established studies have shown that metastatic WM1158 can invade via this amoeboid phenotype (**Grahovac J et al., 2013**). This thesis also indirectly establishes that TGFβ is also released during therapy-induced cell stress cocultures. Thus, there is a need to investigate how melanoma responses to TGFβ shift from the untreated to therapeutic situation.

3.3.3 Alternative TGFβ reprogramming in the stroma

Both normal and cancer-associated fibroblasts appear highly responsive to TGFβ in coculture. This dissertation establishes that robust responses to TGFβ and subsequent CAF activation results in αSMA expression. However, the response is heterogenous with diffuse actin-stress fiber expression with only subpopulations of double positive αSMA^{+} CAFs, a feature which varies between fibroblast cell lines. These double positive cells likely result from a TGFβ dominant response, while $\alpha SMA^{(neg)}$ are activated and maintained by FGF. The bioactive CAF secretome is

contains both growth factors, wherein TGFβ CAFs are pro-fibrotic and associated with invasive SCC, while FGF CAFs are found in non-desmoplastic regions with greater macrophage infiltration (**Bordignon P et al., 2019**). The discrepancy between actin stress fibers and αSMA^{+} could feasibly result from the dual invocation of these pathways. Dominant negative (DN) studies by Bordignon et al., revealed the dynamic reciprocity that exists between TGFβ and FGF signaling in CAFs. DNTGFβR2 resulted in the activation of FGF responses and expression of inflammatory cytokines (CXCL1, CXCL10, CXCL11) and macrophage activation associated with loss of αSMA. Suppression DNFGFR1 drove CAFs to regain αSMA. This is of particular importance given that astrocytes which are the representation stromal component in brain tissue are shown to recruit $CXCR3^{+}$ melanomas through CXCL10 secretion. Our findings show that infigratinib (FGFRi) indeed drives αSMA and supports a dualism between FGF and TGFβ. Whether dual treatment with FGFRi and TGFβRi suppress αSMA needs to be examined. However, given the striking inhibition of αSMA with TGFβRi alone, combining treatments addresses a separate issue that concerns both fibrosis and inflammation. Our coculture data supports the model wherein therapy-sensitive cells boost αSMA expression in CAFs as αSMA was absent in BRAF and MEK targeted vemurafenib resistant melanomas. This finding was surprising given that A375R cells should remain sensitive to MEK and thus secrete stress-induced signals. The effect of immune inflammation was also absent *in vitro* despite transcriptional indications that MCP1 is highly induced from cell stress. This may be attributed the reductionist nature of these cocultures, thus additional assays in *ex vivo* 3D-skin organ cultures or *in vivo* studies are warranted. However, this may also indicate that immune inflammation is not essential to αSMA CAF activation. However, the data herein shows that drug and dose-dependent effects influence the composition of the drug-

stressed secretome, which has downstream impacts on CAF phenotypes. This led additional studies to characterize how activated CAFs impact progression.

3.3.4 The inflamed TME drives melanoma progression

To ascertain the effect of CAFs on progression, modified cocultures with diminished cell-cell contacts were utilized. CAFs had marked impact in promoting the invasive outgrowth of melanoma while the normal counterparts were moderately suppressive for 72hrs. The presence of CAFs from a young patient trended with a lipopolysaccharide/EFG inflammatory stimulus. Aged CAFs had an even greater effect in driving metastatic WM1158 outgrowth. Aged CAFs are reported to increase melanoma invasion and resistance through modified WNT/β-catenin and sFRP2 signaling that ultimately confers greater resistance to reactive oxygen species. Drug sensitivity was restored in melanoma after knockdown of sFRP2, which diminished spheroid expansion *in vitro* and melanoma metastasis *in vivo* (**Kaur S et al., 2015**). Given that MEKi had been shown to drive inflammation of αSMA CAFs *in vitro*, selumetinib was added to these cocultures. This was hypothesized to drive spheroid release of TGFβ and CAF activation, however, MEKi resulted in smaller spheroids and reduced invasion. Despite limited collective outgrowth of the spheroid mass, individual single cells were still invasive. Of more interest was evidence of MEK induced single cell invasion of A375 which routinely invade as a collective mass in the absence of drug stress. The single cells may therefore be more sensitive to TGFβ, suggesting plasticity within cell lines. TGFβ responsive squamous cell carcinomas (SCC) localize to the tumor-stroma interface in mice (**Oshimiri N et al., 2015**), while activated SMAD in complex with transcription factor AP1 (c-JUN, FOS) have been shown to impact ECM proteolysis (Qing J **et al., 2000**). Thus, studies investigating the transcriptional status of highly invasive melanomas during

drug stress may provide insight and targets to blunt invasion. Moreover, the lack of CAF support during drug stress may also be attributed to the temporal nature of this assay, as multiple *in vivo* co-implantation xenograft studies have shown CAFs to promote progression. Interestingly, modified CAF responses may also tie back into immune inflammation in supporting a fibrotic TME. Thus, the seeming abject response of immune cells in coculture could be both contextual and require a longer time scale leading the pro-tumorigenic macrophages. Therefore, additional studies investigating whether CAFs can acquire an immunosuppressive phenotype *in vitro* within the context of melanoma and immune cells is of relevance. This is particularly salient as CAFs derived from patient tumors are reported to lose immunosuppressive genetic signatures in vitro (**Tirosh I et al., 2016).** Thus, 3D-skin organs were used to develop more representational microenvironments in which the bioactive ECM could be probed.

Guided by the *in vivo* studies showing peri-tumor enrichment of αSMA in tenascin-C rich micrometastatic melanomas, we focused on tumor-ECM molecules as mediators of progression. The data leading up to this study had established an essential role for TGFβ in driving CAF activation, however the bioactive secretomes only provide partial insight into how inflammation drives metastatic melanoma. The ECM is an essential mediator of advancing melanoma and our prior studies had established a role of TNC in this process. Tumor-ECM and inflammatory TNC resulted in increased WM1158 invasion into collagen-TNC gels. Conversely, invasion was blunted in anti-invasive DCN collagen gels. Titrating these 'stop-or-go' ECM signals revealed that these representative normal and CAF associated ECM molecules may be therapeutically beneficial. Furthermore, these findings aid in explaining how normal fibroblasts suppress advancing melanoma, while CAFs support progression. TNC and DCN can bind and signal through EGFR with opposite outcomes. Select Epidermal Growth Factor-like (EGFL) repeats of TNC have been

shown to bind to the EGF binding pocket of EGFR (**Iyer et al., 2007**), while the DCN binding site in EGFR partially overlaps but is distinct from the EGF binding epitope (**Santra M et al., 2002**).

We have previously shown that EGFL repeats of TNC promote melanoma cell invasiveness by increasing the ROCK activity and myosin-light chain phosphorylation in migrating cells, which leads to a shift in mode of motility from mesenchymal to amoeboid and allows greater flexibility in 3D microenvironment (**Grahovac J et al., 2013**). Decorin could simply prevent this effect by competing for EGFR given that it has higher affinity for the receptor (**Csordás G et al. 2000**). Alternatively, DCN could signal inhibition of migration through other receptors such as c-Met, PDGFR or IGF-1R (**Grahovac J et al., 2014**), all of which can independently influence melanoma invasiveness. DCN and TNC both bind Toll-like receptor 4, with seemingly opposite effects (**Merline R et al., 2011; Midwood K et al., 2009**). TNC binding to TLR4 was shown to drive pro-tumorigenic M2 macrophage polarization (**Piccinini AM et al., 2016**) and revealed by quantitative proteomics as a potential signature of fibrosis and lung adenocarcinoma progression (**Gocheva V et al., 2017**). Therefore, a natural moiety present in the skin, DCN is an attractive candidate for the development of anti-melanoma therapy. DCN targets multiple kinase receptors involved in cancer promotion in addition to EGFR (**Csordás G et al. 2000; Iozzo RV et al., 2011)** and DCN gene delivery has already been shown to retard the growth of human tumors in immunocompromised animals (**Reed CC et al., 2002**) Thus, methods to restore normal ECM in place of tumor ECM may have a profound impact in limiting the dissemination of melanoma. However, restoration of normal ECM will likely need to proceed through the reprogramming of CAFs. Therefore, targeting molecules such as TGFβ to eliminate the TME will be as essential as targeting the tumor.

4.0 Potential Therapeutic Strategies: Targeting TGFβ and the inflamed TME

The network of signals relayed between the tumor and stroma is both vast and intricate. This thesis has identified an underappreciated role for the off-target effect of TGFβ release into the TME during treatment. Transforming growth factor-β is secreted by stromal, immune and tumor cells and can bind ECM residing in a latent form. However, this cytokine and the ECM to which its binds evolve with the TME leading to tumor inhibiting effects that transition to promote tumors in advanced stages. Thus, targeting TGFβ, or cellular sources, in addition to the ECM that sequesters this protein, may have potential far-reaching therapeutic benefits in melanoma and metastatic disease.

4.1 TGFβ regulatory and targetable functions

There is still work underway to tease out mechanisms by which TGFβ mediates progression in melanoma while educating CAFs to reorganize and signal from the tumor ECM. The thesis shows that TGFβ is secreted into the TME to circumvent growth suppression and activate CAFs that potentially inhibit immunogenic cell death (ICD). Here, the bulk of secreted TGFβ was shown to result from drug-induced stress. Targeted small molecules (BRAF, EGFR and MEKi) used in the treatment of melanoma drove TGFβ release. However, mouse models of colorectal cancer (CRC) experimentally identified stromal TGFβ as a stimulator of CAF derived IL-11 that promoted CRC survival and metastasis to liver (**Calon A et al. 2012**). The release of TGFβ also acts to directly inhibit CD8 T-cell cytotoxicity (**Thomas DA et al., 2005**). In addition, TGFβ also

prevents tumor-infiltrating macrophages and neutrophils by promoting immunosuppressive activation (**Novitskiy SV et al., 2012**) (**Figure 28-1**).

This has spurred the development of inhibitors of TGFβ targeted to TGFβ1, TGFβ2, TGFβ3, or type I and II TGFβR (**Table 1**). However, the pleiotropic signaling capacities of these molecules and downstream signaling cascades have made TGFβ a challenging target (**Demaria O et al., 2019**). Many of these strategies involve direct inhibition via TGFβRI antagonists or sequestration of TGFβ via bispecific ecto-domains and checkpoint blockade. This dissertation tests

Table 1: Current Therapeutics against TGFβ (2020)

Biologics			
Drug	**Phase**	**Manufacturer**	**Format**
Bintrafusp-alfa/ M7824	Phase 2/3	Merck, GSK	Bifunctional "TRAP" Fusion Protein
SHR-1701	Phase 1	Jiangsu HengRui Medicine	Bispecific Fusion Protein
SAR439459	Phase 1	Sanofi	Monoclonal Ab
NIS793	Phase 1	Novartis	Monoclonal Ab

Small Molecule Inhibitors			
BMS-986416	Phase 1	Bristol-Meyers Squibb	Small molecule, TGFβ1/TGFβ3
Vactosertib	Phase ½	MedPacto	Small molecule, TGFβR1
Galunisertib	Phase 2	Eli Lilly and Company	Small molecule, TGFβR1
LY3200882	Phase 2	Eli Lilly and Company	Small molecule, TGFβR1
PF-06952229	Phase 2	Pfizer	Small molecule, TGFβR1

the use of vactosertib (TEW-7197) in combination with selumetinib (MEKi) *in vitro* and i*n vivo* to disrupt stromal activation. Blockade of TGFβRI is hypothesized to target the stroma which acts as a TGFβ sink, given that TGFβ autocrine signaling was not observed in melanoma. Disruption of TGFβ in this way would reduce melanoma EMT and plasticity that drives transition between mesenchymal and amoeboid invasion (**Figure 28-2**). Alternative strategies targeting TGFβ directly using small molecules or biologics such as TGFβR-ectodomain "traps" are currently the

early phases of development (**Table 2**). However, non-specific targeting of TGFβ1/ TGFβ3 will be challenging for these therapies given the varied tissue-specific expression and contexts in which Individual TGFβ molecules signal. Conversely, targeted bispecific PD-L1-TGFβ fusion proteins hold promise to redirect progression while inducing an immunogenic response (**Figure 28-4**). However, these early phase clinical studies vs. monotherapy pembrolizumab are currently under investigation for non-small cell lung cancer (NSCLC) with high PD-L1 expression (NCT0331706). These bispecific approaches are reliant on the sensitivity of immune cells to home to tumors and detect differentially and sensitively detect anti-PD-L1 on tumors.

This dissertation presents preliminary evidence showing that acquired resistance to vemurafenib (PLX4032) results in a drug-specific reduction in PD-L1 expression that correlates with treated cell lines and tumors (**Figure 29A-B**). Thus, bispecific therapies that exhibit efficacy in PD-L1 positive tumors may not lead to the destruction of proliferating resistant melanomas that PD-L1 negative and immunosilent. Therefore, there is virtue in utilizing either monoclonal TGFβR antibodies and/-or small molecule TGFβRI/II antagonists. Targeting the receptor as opposed to TGFβ would be expected to have reduced off-target cytotoxicity due to the fine-tuned intracellular regulation of TGFβRI/II-SMAD (**Figure 28-4**). Moreover, this dissertation has shown that CAFs respond rapidly to TGFβ1, suggesting that these cells readily express TGFβRI/II in comparison to uninflamed fibroblasts. However, strategies would benefit from the characterization of TGFβRI/II expression in CAFs and normal fibroblasts and cancers. Imaging combined with flow cytometric analysis of fixed cells could be used to experimentally determine the absolute number TGFβRI/II molecules during activation. Such data would help in elucidating the levels of TGFβ-TGFβRI/II signaling that coincide with evidence of drug resistance. This detail would help to explain clinical

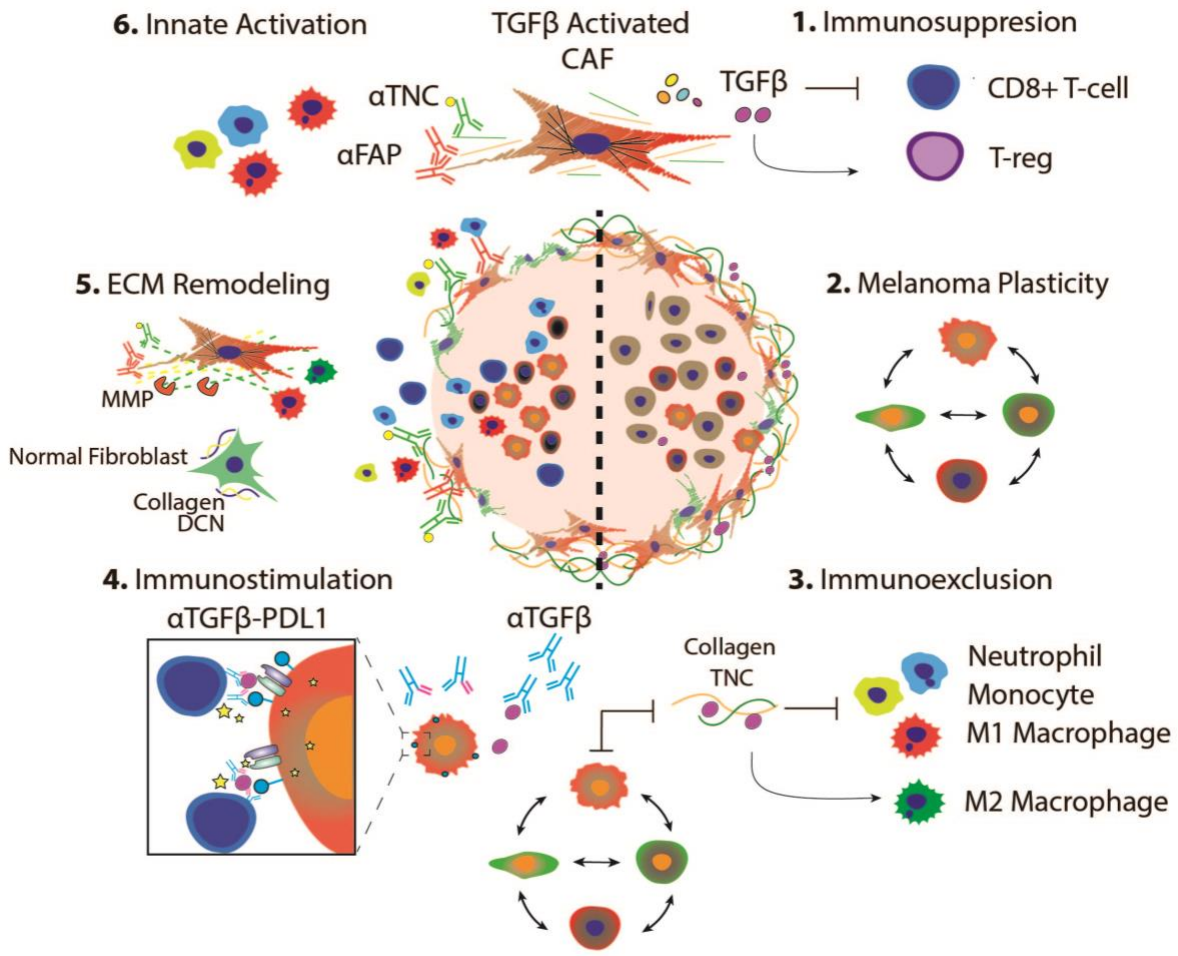

Figure 28: Current and proposed strategies to target TGFβ and the TME
The bioactive TME and drug stressed TME releases multiple cytokines to support tumor expansion and progression. **(1.)** Stromal-derived transforming growth factor-β drives immunosuppressive CAF activation and T-regulatory cells while directly inhibiting $CD8^+$ T-effector cells. **(2.)** Transforming growth factor-β also drives stress and apoptosis early in melanoma, while resistant clones respond by undergoing EMT, shifting between mesenchymal (left) and amoeboid (right), to escape immunosurveillance and form metastases. **(3.)** Pro-fibrotic TGFβ-activated CAFs synthesize ECM that captures TGFβ and excludes immune intravasation, while components such as TNC signal to innate-TLR4 receptors to drive M2-protumorigenic activation. **(LEFT) Therapies designed to suppress TGFβ and progression. (4.)** Multiple immunotherapies and inhibitors of TGFβ/TGFβR are under investigation to halt the TME and drive immunostimulation. Both TGFβ/TGFβR monotherapies and bispecific TGFβ-checkpoint therapies are designed to shut down dysregulated TGFβ signaling and target cancers (melanoma) for adaptive destruction. **5.** Blockade of TGFβ drives tumor regression through stalled production of fibrotic matrix allowing immune penetration and matrix remodeling. **6.** Strategies to target CAFs for innate destruction include fibroblast associated protein (FAP) or monoclonal targeting of aberrant matrix such as TNC.

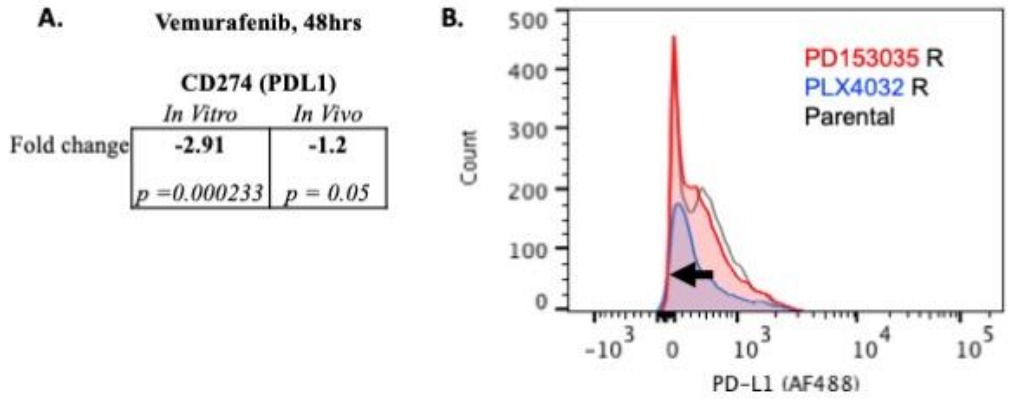

Figure 29: Melanoma resistance to small molecules impacts immunotherpeutic targets

(A) RNA-seq analysis to validate small molecule resistance in melanoma cell lines and xenograft tumors correlates with a reduction in PD-L1 expression (GSE64671). **(B)** Flow cytometry analysis shows a reduction in PD-L1 expression for vemurafenib (BRAFi, PLX4032) resistant A375 melanomas that is not observed for EGFRi (PD153035) resistant clones or parental lines. Data are displayed as geometric mean fluorescent intensity (n=3, technical replicates).

findings such as the efficacy of atezolizumab (PD-L1 monotherapy), which was proportionately associated with TGFβ activity in the stroma (**Mariathasan S et al., 2018**).

Regardless, neutralization of TGFβ signaling in the stroma in combination with immunotherapy or checkpoint inhibitors may prove less cytotoxic than inhibitors of TGFβR. However, our preliminary studies in mouse models show high tolerance no observable cytotoxicity for vactosertib (5mg/kg, ~5mM) in combination with MEK inhibition. This is of particular relevance given that vactosertib effectively blunted TGFβ-stress induction of stromal activation at 1μM in cell culture (**Figure 30**). Interestingly, galunisertib (LY2157299) and anti-PD-1 have shown regression in engineered PDAC mouse models (**Principe DR et al., 2019**). However,

studies combining MEK and TGFβR inhibition are limited, and there are no current published studies or clinical trials for TGFβR inhibition in melanoma (**Table 2**). Therefore, this dissertation

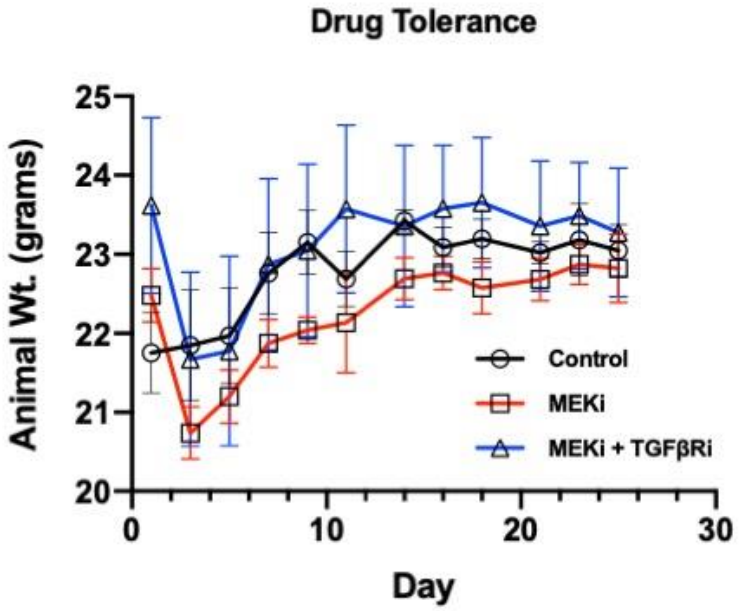

Figure 30: Animal tolerance to selumetinib and combination vactosertib therapies

Animals bearing human metastatic melanoma xenografts were treated with control, MEK inhibitor Selumetinib (10mg/kg) alone or in combination with TGFβRi Vactosertib, TEW7197 (5mg/kg) for 4 weeks following a 7-day tumor establishment period. (n=4 animals per group). Data are presented as mean ± s.e.m.

is the first to test the effects of limiting TGFβ during the treatment and progression of melanoma. This dissertation proposes that the short-term stress responses in melanoma promote TGFβ-driven inflammation of CAFs, aberrant ECM remodeling, and progression to metastatic melanoma. Thus, this investigation dually concerned with eliminating tumor progression and in therapeutically preventing inflammation in the tumor stroma via suppression of TGFβ and tumor promoting ECM.

Table 2: Clinical Trials Targeting TGFβ Activation in Cancer

Clinical Trials Targeting TGFβ Activation						
Status	**Phase**	**NCT Number**	**Interventions**	**Acronym**	**Conditions**	**Results**
Not yet recruiting	Phase I-II	NCT04031872	LY3200882	EORTC1615	CRC	NR
Recruiting	Phase I	NCT03579472	Anti-PD-L1+TGFβRII Fusion	M7824+Eribulin Mesylate	Triple NEG Breast	NR
Recruiting	Phase I-II	NCT03436563	Anti-PD-L1+TGFβRII Fusion	M7824	CRC, Stage IV	NR
Recruiting	Phase I	NCT04291079	SRK-181|Anti-PD-L1		ADV-MET Solid Tumor	NR
Recruiting	Phase I-II	NCT03666832	Vactosertib (TEW-7197)	MP-PDAC-01	MET Pancreatic	NR
Recruiting	Phase I-II	NCT03732274	Vactosertib (TEW-7197)		NSCLC Metastatic	NR
Completed	Phase I	NCT02160106	Vactosertib (TEW-7197)		ADV Solid Tumors	NR
Completed	Phase II	NCT01401062	Fresolimumab+Radiation		MET Breast	R
Withdrawn	Phase I-II	NCT03470350	Galunisertib	EORTC1615	CRC	NR
Suspended	Phase I	NCT02672475	Galunisertib+Paclitaxel		Triple Negative Breast	NR
Completed	Phase I	NCT02734160	Galunisertib+Durvalumab		MET Pancreatic	NR
Recruiting	Phase I-II	NCT03893695	GT90001+Nivolumab		MET HCC	NR

4.2 Overcoming tumor ECM and immunosuppression

Future studies should investigate how CAFs are reprogramed in the presence of stress induced TGFβ. The coculture systems have been utilized to isolate RNA from normal and activated CAFs. Analysis of genes listed in appendix E in addition to cell-adhesion molecule arrays will provide beneficial insight into key ECM proteins that are activated in response to melanoma stress.

These proteins are presumed to look similar those that are expressed early in wound healing. Many of these proteins are associated with inflammation, or fibrosis if chronically expressed. Experimental evidence of this has been established in lung adenocarcinoma, wherein human tissues were proteomically profiled. The authors found that TNC was upregulated in fibrosis and metastasis (**Gocheva V et al., 2017**).

Another study implicated chemotherapy induced stress in breast cancer as an activator of c-Jun (JNK) which lead to TNC and SPP1 activation. Inhibition of c-JUN (JNK) with JNK inhibitors abrogated these ECM molecules and restored sensitivity to chemotherapy (**Insua-Rodríguez J et al., 2018**). Pathologically inflamed ECM is paradoxically linked to TGFβ signaling in fibroblasts, which are postulated to create collagen rich peri-tumor stroma that prevents both drug and immune cell penetration (**Figure 28-3**). This dissertation identified the colocalization of αSMA stroma flanking TNC rich micrometastatic melanoma nodules in the livers of xenografted animals. Future studies would benefit from additional staining of tumor sections to correlate collagen expression with CD68 macrophage and $CD8^+$ infiltrate in untreated and treated groups. The TGFβ shaped ECM fortress surrounding metastatic urothelial cancer has been credited with preventing T-cell infiltration and subsequent failure of PD-L1 atezolizumab. This occurred in spite of evidence pointing to inflamed melanomas are most responsive to checkpoint blockade (**Tumeh PC et al., 2014**). Combined check point inhibition and TGFβ blocking antibodies suppressed CAF signaling and resulted in intra-tumoral T-cell penetration and tumor regression (**Mariathasan S et al. 2018**). A similar outcome in mouse models of metastatic CRC found that stromal TGFβ limited T-cell and differentiation of effector T_H1 cells. However, targeting TGFβR1 with galunisertib ablated the immunosuppressive stroma, and enabled $PDL1^+$ immune infiltration into established liver metastases and restored anti-PDL1 ICD (**Tauriello DVF et al., 2018**) (**Figure 28-5**).

Prompted by this, we established an *in vivo* study examining the effects of combined inhibition of MEK in patient 12-293 metastatic melanoma and vactosertib mediated suppression of TGFβRI/ALK4/5 to suppress therapy induced CAF activation. Preliminary evidence from this study showed inhibition of metastases to the liver and objectively reduced burden to the spleen injection site. Thus, future studies will focus on examining tumor reduction and cell death with respect to the ECM and αSMA stroma. Particular focus should be given to TNC, where this molecule is expressed (intratumorally vs. invasive edge), and whether DCN exhibits mutually exclusive expression.

Tenascin-C is also highly expressed in metastatic melanoma and is seemingly an attractive signature or target (**Figure 28-6**, **Table 3**). However, there is uncertainty regarding where the TNC epitope of Iodine (I) I-131-monoclonal 816C antibody is expressed in tissues. Therefore, this antibody does pose toxicity issues if it were to bind TNC in non-involved or fibrotic tissues. In these non-specific sites, this antibody could potentially activate or exacerbate pre-existing inflammation. However, there may be opportunity to exploit these antibodies as bispecific(s) with EGFR or PD-LI, or TGFβR moieties. However, designing these may be challenging. Thus, immediate head-to-head tests comparing mono-vs.-combination therapies cetuximab (anti-EGFR) and anti-EGFL TNC antibodies could prove useful in metastatic melanoma. These approaches could be elaborated by inclusion of TGFβI/II small molecule inhibitors or therapeutic antibodies. This work is pivotal as there is currently no evidence on whether antibody dependent cell-mediated cytotoxicity (ADCC) drives cell-stress. However, targeting TNC will remain challenging nonetheless, as all trials were stopped at Phase II with not reported results. Therefore, the best strategy is likely one that suppresses inflammatory ECM molecules through the suppression of the CAFs, which are the source.

This dissertation aims to expand the known repertoire of ECM molecules that are differentially expressed in response to melanoma TGFβ and how this transcriptional landscape is changed during combined MEK/TGFβR in vitro inhibition. Our preliminary data shows that this is well tolerated and effective at preventing metastasis in mouse models. However, there may be additional opportunities to reinvigorate the use of TNC targeted monotherapies, by combining these epitopes with antibodies against fibroblast associated protein (FAP). Combined targeting in this manner is expected to prove more efficacious in the treatment and destruction of CAFs. This therapy may even have hitherto unappreciated use in treating TNC positive fibrotic diseases. Beyond these conventional approaches, TNC monotherapies could also be used in the generation of neo-antigens for use in chimeric antigen receptor (CAR) T-cell therapies. There already exists CAR-FAP T-cells that have been efficacious in the treatment of cancers. Moreover, circulating T-cells with antigens for early response proteins such as TNC which are vital to establishing micrometastases disease, may prove highly useful in the management of metastatic melanoma, both in limiting residual disease and by abscopal effect. However, the studies presented in this section would be foundational for any future approaches regarding bispecific targeting or T cell-based therapy. Ultimately, there is great promise in limiting the inflammation of the TME while simultaneously targeting melanoma and cancers to drive apoptosis.

Table 3: Clinical Trials Targeting TNC Activation in Cancer

Clinical Trials Targeting TNC Activation						
Status	**Phases**	**NCT Number**	**Interventions**	**Acronym**	**Conditions**	**Results**
Terminated	Phase I	NCT02602067	I-131-Tenatumomab	Tenatumomab	TNC^{+} Cancers	NR
Terminated	Phase III	NCT00615186	Neuradiab + Radiation + Temozolomide	Glass-Art	GBM	NR
Completed	Phase I	NCT00002753	Radiation+I-131 Mab 81C6		Brain/CNS	NR
Completed	Phase I-II	NCT00003478	Surgery+I-131 Mab 81C6		Brain/CNS	NR
Completed	Phase I-II	NCT00002752	Surgery+ I-131 Mab 81C6		Brain/CNS	NR
Completed	Phase I	NCT00003484	Carmustine+Radiation+I-131 Mab 81C6	Irinotecan HCl	Brain/CNS	NR
Unknown status	Phase II	NCT00906516	Neuradiab+Bevacizumab	(Avastin)	Brain/CNS	NR

Appendix A – The great escape: how metastases or melanoma, and other carcinomas, avoid elimination.

Text from this chapter appears in *Experimental Biology and Medicine (Maywood)*. 2019. 243; 17-18:1245-1255 as a minireview on Anatomy/Pathology.

Synopsis

Cancer mortality ensues from metastatic growths. Cancers use two strategies to allow for this unrelenting expansion. The first way is that early metastases are often cryptic or dormant, being invisible to both innate suppressive actions and undetected clinically. Second, both the micrometastases and later clinically lethal growths are resistant to therapies, whether standard chemotherapies, targeted biologics, or even immunotherapies. These two modes of resistance necessitate new approaches to treatments if we are to eliminate mortality from solid tumors. However, to develop such therapeutic strategies, we first need to better understand the cellular behaviors and molecular events that enable the resistances. Herein, we present a comprehensive model of changing methods of avoidance and resistance that occur during tumor progression, and doubly confound treatment by mixing survival strategies throughout the continuum creating moving targets. Melanoma is presented as the model cancer, as it is being targeted by all three types of agents for disseminated disease, with breast and prostate cancer as two other key carcinomas.

Appendix A.1 Introduction

Metastatic progression of solid tumors is the harbinger of most morbidity and mortality from solid tumors. The initial primary tumor growth is most always capable of being eliminated by surgical excision or radiological ablation. While this may lead to real morbidity from the surgery and its sequelae, and the unusual death, the outcomes are increasingly curative as we appear to catch many at their earliest stages. Over 80% of melanomas and breast and prostate cancers are eliminated in this manner. However, if the tumor has already disseminated, the long-term outlook becomes bleaker with five-and-ten year survivals falling dramatically; for melanoma, fewer than a third of patients survive past a half-dozen years.

The issues that confront the survivors of the primary lesions are that the metastases are not only not amenable to removal, due to wide dissemination, but also that these cancer cells are generally more resistant to cancer-targeting agents. This is seen most clearly with neo-adjuvant breast cancer treatments that will shrink the breast nodule, even leading to a complete pathological response (no cancer cells detectable) upon subsequent lumpectomy, while not significantly impacting the overall survival (1-3). At least a subset of the disseminated cells presents distinct properties from the primary tumor cells in terms of therapeutic responsiveness. As this is seen even with therapy naïve cancers, it is unlikely that this is due to genetic mutations, but rather is micro-environmentally imprinted on these cells in ectopic sites.

To overcome such resistance to therapy, we need to understand the phenotypic and cellular changes that cells undergo during progression. Initial studies have begun to dissect such switches and decipher their impacts on therapeutic responsiveness. Herein, we propose a unified model for tumor progression linking the micro-environmental induction of phenotypic switches with

susceptibility to therapeutic approaches. This provides for testable hypotheses and pathways to re-sensitize disseminated tumor cells to accepted therapies.

Appendix A.2 The Metastatic Cascade

Dissemination from the primary site relies on the ability of tumor cells to survive and grow in a metastatic site and requires many changes to adapt to the tissue-specific environment unique to each organ (4). Tumor metastasis, even more than carcinogenesis, involves both the carcinoma cell and the host environment (Figure 1). Dissemination most likely requires distinct cancer phenotypic switching. Initially, a cancer-associated epithelial-mesenchymal transition-like plasticity (cEMT) allows for the autocrine signaling and loss of cell-cell constraints that promotes escape from the primary site (5, 6). It should be noted that in referring to cells as epithelial or mesenchymal, we are denoting the presence or absence of cell surface E-cadherin, loss of cell-cell cohesion, and shape shift towards fibroblastoid without epithelial cell polarity; this is not a true phenotypic switch at the full transcriptome level (7). This phenotypic plasticity, in part driven by the now-enabled autocrine growth factor signaling through the EGF receptor (8, 9) and downstream involving the Met receptor for HGF (10). This is further augmented by signaling from an altered localized micro-environment, both the matrix and the cells. This resembles a wound environment, with activated fibroblasts (CAF, cancer-associated fibroblasts) and an immature reparative matrix (11-14). The productive fibroblasts secrete signals not only for the cancer cells

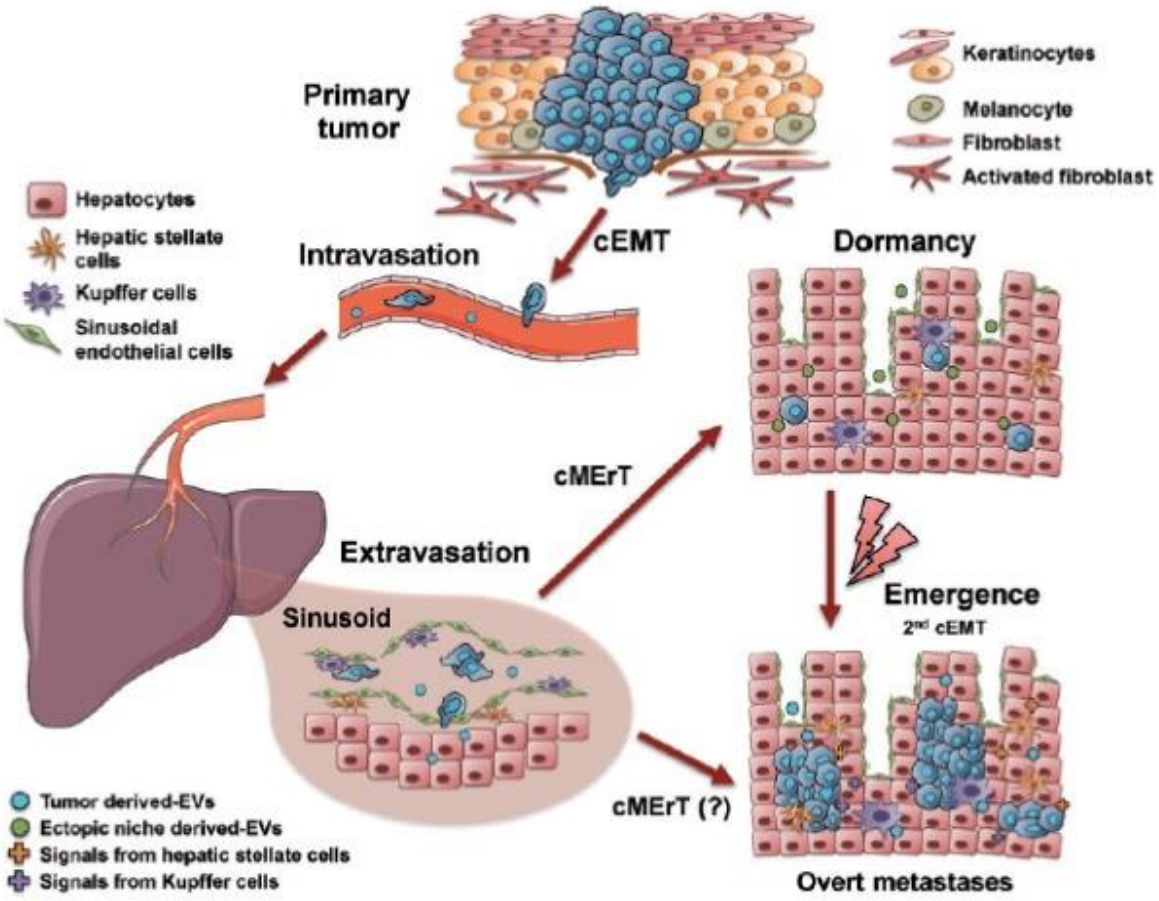

Figure 31: Metastatic Cascade to Liver

Schematic of metastasis with postulated phenotypes that confer resistance or sensitivity to therapies. Disseminating carcinoma cells must acquire mesenchymal-like migratory properties to escape the primary locale (the cEMT). Transit through vascular conduits to sites of metastasis are fraught with challenges with most tumor cells not surviving but are sufficiently transient to not represent a target for therapy. At the metastatic site, cells must survive apoptotic cytokines initially and chemotherapy later; this is accomplished by a reversion to a more epithelial phenotype and expression of E-cadherin (cMErT) that in turn provides for the chemoresistance and immune silence [PD-L1-negative], driven at least in part by host organ extracellular vesicles (Green bubbles). Cells emerge from dormancy due to inflammatory stimuli (lightning bolts) to form aggressive, lethal metastatic nodules with re-acquisition of mesenchymal-like behaviors and cause the organ to secrete factors with secondary metastases also ensuing. (adapted from (58)).

but also to attract immune cells that further promote invasion and dissemination (15, 16). The combination of a mesenchymal, highly motile and physically plastic cancer cells with a disrupted pliant matrix allows for invasion through these barriers, whether the limiting basement membranes and outer muscular capsules in carcinomas or the thicker dermis in melanomas.

After transiting these barriers, the cancer cells must survive travel in a flowing conduit to metastasize. Whether the intravasation occurs in the hematogenous or lymph vasculature, the final extravasation is occurs from the hematogenous capillary bed (lymph node seeding is a separate category of dissemination from ectopic organ metastasis). While the processes of this transit and survival in a turbulent flow are complex, this short event is neither a reservoir of tumor cells nor an opportunity for treatment and will not be discussed further.

At the other end of dissemination, the greatest challenge for metastatic seeding is integration into the ectopic metastatic tumor microenvironment despite the lack of a supportive environment and presence of pro-death signals from a local inflammatory response. Interestingly, metastases of breast and other carcinomas often express E-cadherin (9, 17-19), whose initial loss is the hallmark of cEMT and correlates strongly with dissemination (20), even if expression of mesenchymal markers are absent (21). This reversion to an epithelial phenotype is secondary to loss of mechanisms that suppress E-cadherin expression such as loss of promoter methylation (17) and Kaiso binding to the promoter (22-24), and abrogation of EGF receptor signaling that downregulates E-cadherin (8). We have reported that carcinomas and melanomas re-express E-cadherin in metastatic tissues such as liver, lung and brain (7), while others have reported such in bone marrow metastases (18, 19, 25), but not in lymph nodes (data not shown). Other epithelial markers including connexins are similarly upregulated in the metastatic niche (20). This reversion of cEMT or a cancer-associated mesenchymal-epithelial (reverting) transition (cMErT) is only

partial to the same extent as the initial cEMT is also partial (7), suggesting a metastable or plastic situation that allows for outgrowth upon a second cEMT (6).

A portion of these disseminated cells can enter a period of dormancy to later emerge as aggressive metastatic tumors. This ‘dormancy’ may be quite transient, as in the case of triple negative breast cancers, or extended as in hormone receptor-positive breast cancers (even in excess of two decades before clinically evident recurrence). However, evidence is accumulating that epithelial reversion is a feature of dissemination for most all carcinomas and likely melanomas. The mechanisms underlying dormancy are poorly understood as successful metastatic seeding and dormancy (as opposed to primary escape and dissemination (26)) are rare events (27, 28). This gap in our understanding is due in large part to the absence of tractable experimental systems that can focus on this stage to metastasis, a limitation we will discuss later. Successful ectopic seeding with its epithelial reversion appears to be linked with entry into dormancy. The tumor signals that enable intercalation within a tissue (including extracellular vesicles [EV]) (29-32) leads to reverse signaling (including EV) from the receptive organ that imparts the cMErT and initial dormancy (33, 34).

The state of the tumor cell in dormancy is unknown, with one model positing quiescence versus another of balanced proliferation and death. In silico modeling determined that it is highly unlikely that micrometastases exist in a state of balanced proliferation and death, but rather either grow out or enter quiescence (35, 36). The quiescent dormancy model is supported by findings of suppressive matricellular proteins in the pre-metastatic niche coinciding with failure to establish macrometastases (33, 37). As will be discussed, whether the cells are cycling or quiescent during dormancy would affect their ability to be targeted by cycling-dependent agents such as those

disrupting DNA replication or intermediary signaling pathways for mitogenesis (such as raf and MEK inhibitors in use for melanomas).

This also raises the questions of what signals keep the cells in quiescence, and which 'awaken' these dormant metastases. It appears that the (re-)expression of E-cadherin coincides with entering dormancy (7, 17), but does not dictate quiescence as cells expressing surface E-cadherin demonstrate mitogenesis particularly notable in the early stages of melanoma- and carcinoma-genesis. Importantly, this emergence from dormancy occurs along with a second cEMT shift (7, 38). Initial studies suggest that stressors of the microenvironment, whether inflammatory cytokines or immune activators, can induce cEMT even in well-differentiated carcinoma cells (39-42). This is supported by our finding that stressed endothelial cells activated a emergent or mesenchymal phenotypic shift in an E-cadherin-expressing breast carcinoma cell line via secreted growth factor (39), and work by others implicating a role for endothelium undergoing sprouting as driving tumor emergence (33). Additional signals can derive from activated stellate cells and macrophages to promote this same shift (40, 41). Interestingly, using an ex vivo model for dormancy and emergence, we have reported that outgrowth is reflected in a globally inflamed organ (as denoted by increased levels of cytokines, chemokines and growth factors) while dormancy occurs in the setting of an organ that is 'quieter' than normal homeostasis (43, 44). This evidence that emergence results from, or at least along with, chronic inflammatory activity, along with recruitment of immune cells should have implications for the sensitivity of such emergent growths to immunotherapies.

Appendix A.3 Investigative Models

The study of therapeutic resistance is hindered by model systems that truly reflect either the development of metastases or the treatment of such in humans. Examination of patient specimens is capable of capturing the heterogeneity both between and within patients but suffers from a number of limitations. The mains ones are (a) static sampling and thus not open to determining cancer plasticity, (b) inability to evaluate the earliest stages which are undetectable, (c) interventions limited to singular regimens, and (d) selection bias. To overcome these, a number of models have been developed to isolate specific aspects of metastasis and therapy, each with their own benefits and limitations.

Appendix A.4 'Spontaneous' rodent metastasis

There are a number of variations of these models. The basic concept involves tumors that develop in mice usually due to genetic engineering to express a specific oncogene in the target tissue; examples include the V600E melanoma (45) and various hormone-driven oncogenes in breast cancer (46, 47) or prostate cancer (48, 49). Chemically induced tumors include those of the liver and other organs. In a few cases, the tumor has developed spontaneously in a particular strain, such as the B16 melanoma, and then is re-introduced into syngeneic animals. For the genetically engineered models, the tumor biology follows from the specific mutations made, and thus only reflects a subset of patients.

These whole animal models benefit from being in intact (i.e. immunocompetent) animals and capturing carcinogenesis and progression. The endogenous nature, or syngeneic aspect when

transplanted, allows for a complete immune system response, including acquired immunity, even if the murine immune systems is not directly replicative of the human situation. A major limitation is that these tumors often do not metastasize at high frequency or predictably. This may be overcome by isolating tumor cells from metastatic target organs and re-introducing them orthotopically with serial enrichment yielding more pliant models.

Appendix A.5 'Inoculated' rodent models

Rodents are used as the hosts of transplanted tumor cells for two reasons in metastasis research. In the first, it is to directly inoculate tumors into ectopic tissues, providing for grafting of tumor cells in organs of metastasis; this is most often published as injection into the tail vein to obtain tumors in lungs, and into the left ventricle to obtain tumor cells disseminated widely with a high predilection to bone. In the second aspect, it is to seed human tumors to study the heterogeneity of human specimens or the de novo nature of carcinogenesis unique to humans; these tumor xenografts can be from cell lines or directed human specimens (PDX) either orthotopically or ectopically. Of course, these two aspects are not exclusive, with human tumor cells often being targeted directly to metastatic organs.

The advantage of the direct inoculation into ectopic organs is that the tumor reaches the ectopic tissue at predictable rate and time, enabling tracking of specific stages of metastases. This has been used to study both longer term outgrowth and treatment and even the initial minutes to hours of seeding. In a seminal study, B16 melanoma cells were introduced via the portal vein into the liver to track their fate over the ensuing hours to days to weeks; this demonstrated a high rate of extravasation but <1% long-term outgrowth rate even for this clonally-selected highly

aggressive tumor (27). Such approaches have been extended to metastatic seeding in the brain (28).

Despite these advantages, this 'directed metastasis' approach opens the question to whether the normal pathological processes are overwhelmed and thus the results are skewed. While the very low efficiency, often on the order of 0.1% of inoculated cells resulting in macrometastases, reflects the likely human situation, the rapid appearance of the macrometastases belies the clinical experience wherein even 'rapid' recurrences require months to years before reaching similar sizes. It is quite likely that the large bolus of extravasating cells overwhelms the pathophysiological responses that drive the epithelial reversion and dormancy noted when limiting numbers of cells are introduced. The quantitative nature of this balance is noted in tumor cells producing greater numbers of EV than the host parenchymal cells, but with this being counterbalanced by the numerically vaster numbers of host cells upon single cell seeding (34). Similarly, the growth factor-rich milieu of tumor cells may also overwhelm the death signals from the innate immune response to foreign bodies/cells, enabling greater survival of mesenchymal aggressive tumors. This is important in studying resistance to therapy if the surviving and outgrowing 'metastatic' tumor cells have not upregulated key molecules and behaviors that normally function to enable metastatic seeding, and thus would appear as falsely sensitive growths.

Spontaneous metastases from orthotopically placed tumors avoids this 'bolus' effect. The resulting metastases undergo the patho-physiologically relevant phenotypic switches and more likely reflect clinical situation (50). For syngeneic rodent tumors, this includes the input from the innate and acquired immune systems (and subsequently allows for examining immune-modulating therapies), but still suffers from the limited diversity of rodent tumors and translatable discrepancies to the human immune system. For human xenografts, this captures the clinical

heterogeneity and can parse out those cells that have metastatic capacity. However, the immune-deficient hosts that are required obviate the input from any acquired immune system inputs or the assessment of such tumor vaccines or lymphocyte-mediated immunotherapy (the immunologic competence or completeness of 'humanized' mice hosts is still uncertain and usually does not match the transplanted immune system with the particularly tumor cells). Even the innate immune response may be limited in these hosts, though the *nu/nu* mouse system appears more robust and replete than the more facile NOD SCID mice (51). The involvement of the immune system inputs in metastasis and therapy are open to question as there are subtle but important differences between rodents and humans; for melanoma in particular this is amplified by the overwhelming predominance of gamma-delta T cells in the skin site of melanoma-genesis and invasion, versus the human situation in which the adaptive alpha-beta T cells are the vast majority in these locales (52).

Appendix A.6 Ex vivo models of metastasis

Ex vivo organotypic models can provide an all-human context as the human microenvironment has species-specific signaling, and many of the newer biologic and immunologic therapies are optimized to work with human sequences. Equally important, this approach can allow for near continuous assessment of the metastatic cascade, with repeated imaging of the same micrometastasis. Sacrificing the entire animal model for a more limited organ tissue allows for such analyses. The details of these different models vary but certain limitations are shared including (a) lack of input (soluble and vesicular signals and hormones) from other

tissues, (b) limited immune system presence and functioning, and (c) time-span limitations of days to weeks.

The simplest replete models are tissue slices, most often of brain or liver, onto which tumor cells can be directly seeded. These allow for limiting the number of cells to reflect the clinical situation and following multiple individual events. As brain and liver are two common organs for metastases and are often the ones of worst prognosis, the clinical relevance of attacking these micrometastases is obvious. Two other commonly involved organs, lung and bone marrow, do not lend themselves to tissue slices due to the physical nature of air/tissue and bone/marrow interfaces, respectively. Compromising the utility of these models is the lack of fluid flow through the tissue that leads to hypoxia and buildup of toxic metabolites that limits the functionality to days.

Recent developments in microphysiological systems (MPS) provide the opportunity to overcome some of these challenges. MPS are complex tissue constructs, generated from individual components (rather than fragments of human tissues), and maintained under engineered conditions that provide for continuous circulation of media, to avoid static conditions that plaque the tissue slices. The cells are either primary human, or more often for ease and reproducibility non-transformed cell lines or differentiated iPSC. The former can be highly limited (except for skin cells), while the latter two groups are not fully mature, functional and differentiated. On a positive note, the media flow can be regulated to deliver nutrients and therapeutic agents in a manner reflecting the human physiology or the pharmacokinetics of drugs. This enables these constructs to function for over a month, a period sufficiently extended to study by drug efficacy and resistance, and secondary challenges (43, 44).

Again here, the most advanced of these MPS are skin, neuropile/brain and liver. While the skin organ cultures have been used to study melanoma invasion (53, 54), the greater potential for

examining the behavior of tumor metastasis lie in the target organs. As these MPSs are nascent, there are few studies investigating metastatic behavior, though even these have led to new insights. It was with an early liver MPS that the tight junctions that metastatic carcinomas can form with parenchymal cells were first reported (55), and that the epithelial reversion was dissected (17, 34, 56). The construction of the current MPS and the complete complement of parenchymal and nonparenchymal cells (including immune cells) is described elsewhere (57, 58). These studies in the liver MPS have recently been used to examine the role of inflammation in driving emergence from dormancy (40, 43, 44), with findings reflecting those suggested by clinical experience and animal models (33, 37).

Appendix A.7 Resistant to therapy

The greatest challenge to treating metastases is that these appear to be inherently harder to kill or control than the primary tumors from which they arise. This is not often able to be determined directly, as primary tumors are mostly found prior to clinically evident metastases and removed. However, in clinical situations where tumors are treated along with synchronous metastases or as neoadjuvant prior to removal, it appears that the disseminated cells are less responsive. A large, early meta-analysis of neo-adjuvant therapy for breast cancer found no increase in overall survival despite strong responsiveness of primary cancers (1). Even when both the breast primary and positive lymph node nodule respond the adjuvant systemic chemotherapy with complete pathological remission, the cure rate is far less than complete (3). These insights suggest a survival advantage for the disseminated cancer cells. Herein, we will provide a model, based on preclinical studies of micrometastases to account for this resistance to therapy.

This discussion will focus on generalized resistance to therapies. This is distinct from selective resistance that often appears in the surviving cells after a particular agent is used and is usually accompanied by a genetic change that may confer survival; this is often selected in preclinical models by repeated exposure to subtotal death challenges. A long-standing well documented situation is the genetic amplification of DHFR (dihydrofolate reductase) to escape killing by methotrexate; a property exploited in genetic engineering of cells. Other situations include target molecule point mutations to render them impervious to the agent (tubulin mutations to render taxanes ineffective), upregulation of P-glycoprotein/MDR1 drug efflux pumps (for many chemotherapies such as doxorubicin), loss of the molecular target of the agent (one example is HER2, accompanied by upregulation of related oncogenes), and second molecule mutations to compensate (this is the mechanism for vemurafenib resistance by commensurable NRAS mutation). While overcoming such specific survival mechanisms are presumed to be critical, the combination approach to most disseminated cancers make these individual changes less likely. The latter points to either a non-genetic adaptation or a protective effect in the disseminated cells, which is the aspect that will be proposed herein.

It has become accepted that the microenvironment contributes to the generalized chemo- and immuno-resistance of metastases (59-62). While much work has focused on specific cancer cell intrinsic mechanisms for resistance to individual agents as noted above, the issue confronting our patients is generalized therapeutic failure. This pan-resistance appears to be due to trophic factors and signals from the micro-environment (61, 63-67) that drive changes in the cancer cells and the microenvironment itself (68). What remains unclear is if a particular tumor is protected by specific factors (e.g. TGFβ production or collagen-mediated suppression), or if there is a progression wherein during the early stages of seeding E-cadherin-mediated signaling comes to

the fore while larger emerging metastases drive stromal reactivity to provide protective factors such as tenascin C (14, 54, 69). As there is little evidence of trophic changes in the earliest single or few cell stage and the carcinoma cell phenotype is plastic (6), we posit that the microenvironment and the cancer changes in response to this drive different levels of therapy resistance at each stage of progression (Appendix A - Figures 2-4).

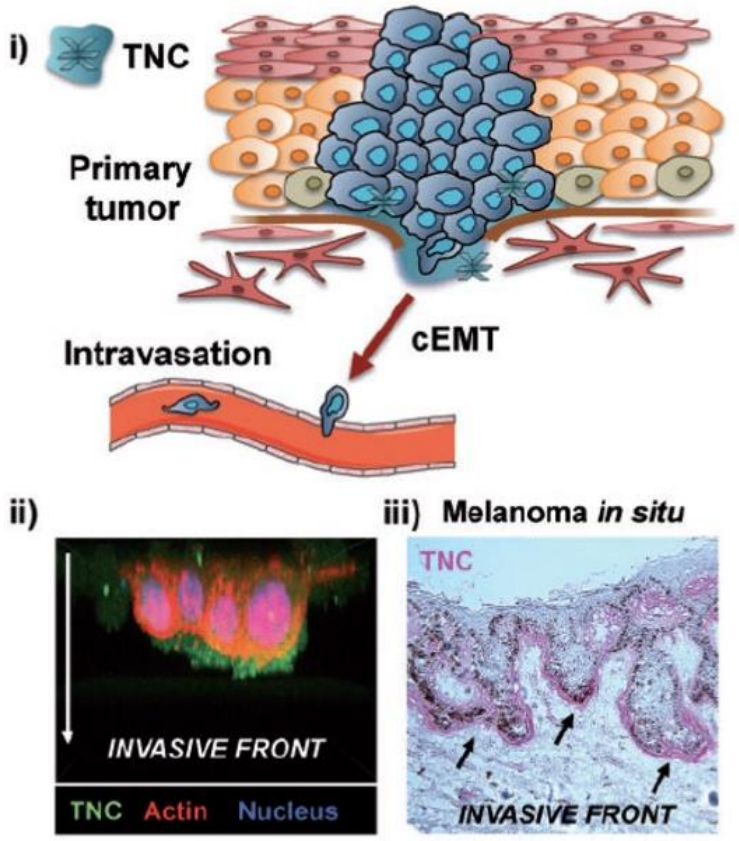

Figure 32: Mechanisms of therapeutic resistance in the primary tumor

i) Escape from the primary tumor site is promoted by the tumor microenvironment being altered with upregulation of motility-promoting matricellular and matrix-associated proteins (*e.g.* TNC). TNC is at the invasive front as demonstrated in ii) an invading melanoma cell lines, and iii) a patient melanoma specimen (adapted from (54)). However, as these cells are highly active, these protections from death are minimal in the pre-disseminated site.

Appendix A.8 (Limited) Resistance in the primary site

The primary tumor site is often the most sensitive to therapies (Appendix A – Figure 2), though the usual approach is to remove surgically, particularly for melanoma, or radiologically in some internal carcinomas. However, in some cancers, neoadjuvant therapy is being used prior to surgical removal. These clinical experiences have shown remarkable responses in primary breast cancers shrinking and disappearing (1, 3) even though the impact on overall survival is small to non-existent. Furthermore, clinical experience with carcinomas with synchronous metastases (and therefore often not surgically resected) often shows a differential responsiveness, as hinted at by the dichotomy of complete pathological response of primary breast cancers to neoadjuvant therapy with limited impact on clinically undetected metastases that drive the overall survival rates. This is noted with both chemotherapies in which metastases persist through treatment (70), and even killing by cells of the innate or acquired immune system (71, 72).

Still, a fraction of the invasive tumor cells is capable of surviving lessened cohesion to cells (and the survival signals from those connections) and transit through an ectopic environment/barrier, the dermis for melanoma. While much of the invasiveness appears to be syncytial, bringing the orthotopic support systems, amoeboid motility of the melanomas allows for separation at the front. In breast cancer it has been noted in experimental systems that disseminating cells break from the nodule to migrate and intravasate separately (73). Both the escape and survival during the process are promoted by the tumor microenvironment being altered with upregulation of matricellular and matrix-associated proteins normally present during wound healing (53, 69). Not only do these proteins promote migration and invasion, but also impart survival advantages (74, 75). However, as these proteins are limited to the leading edge of these invading tumors (54), the protection is likely limited.

Appendix A.9 Avoidance of the micrometastases

Successful seeding of the ectopic site, with less than 1% of the cells that reach to target organ, is the most rate-limiting step in the metastatic cascade (27, 28). Thus, the survival mechanisms are either present in a small fraction of the disseminated cells, or only a limiting number of even clonal cancer cells can undergo changes sufficiently rapidly to survive. We posit that this involves the phenotypic switch that is linked to dormancy (6) (Appendix A – Figure 3).

Disseminated tumor cells face a hostile microenvironment in which the orthotopic matrix and cell composition is lacking, and thus the cell is absent the trophic factors that sustain homeostasis in the original tissue. Cells can overcome this in a number of ways. First, they can produce some of the factors themselves, such as secretion of tenascin C by melanomas (14, 53) and other cancers. In addition, signals from the metastasizing cancer cells alters the host environment to produce some of these 'wound' matrix components (34, 76). A second mode is to switch to a low metabolic state by entering quiescent dormancy; this requires a reversal of the autocrine growth factor signaling that characterizes the initial cEMT and drives proliferation (8, 20). Thus, the successful micrometastases avoid the starvation of loss of trophic factors.

Another challenge is the nonspecific inflammation triggered locally by intravasation and intercalation of the tumor cell into the ectopic parenchyma. This releases both soluble and cellular challenges. Part of the escape from the cellular attack is for the metastatic cells to be immunologically invisible. There is mounting evidence that PD-L1, which imparts aggressiveness to cancer, is downregulated in successful micrometastases of prostate and breast carcinomas (77, 78), and likely also in melanomas (79). Interestingly, this immune escape may be linked to the

cMErT via limited maturation of PD-L1; EGF receptor signaling drives N-glycosylation needed for cell surface presentation of PD-L1 (80), but during cMErT EGFR signaling is limited (8).

Worse than immune-escape, is the situation of paradoxical response of aggressive outgrowth in response to immunotherapies, a situation seen in up to one quarter of patients and termed hyper-progressive disease (81). A recent clinical report has described a cohort of 406 patients having advanced non-small cell lung cancer and treated with PD-1/PD-L1 inhibitors in which 13.8% experienced hyper-progressive disease which was correlated with a significant overall worse survival (3.4 vs. 6.2 months) (82). This situation has also been noted anecdotally in melanoma (83). While the mechanism of this hyper-progressive response to presumed anticancer agents remains unclear, the proposed model suggests a number of testable hypotheses that are concordant with an initial suggestion that activation of the microenvironmental tumor associated macrophages contribute to this perverse outcome (84). This would represent yet another mechanism for escape from treatments.

Successful seeding also likely requires acquisition of positive signals and not just limiting damage. This is likely provided by the E-cadherin signaling upon heterocellular ligandation after cMErT (20). E-cadherin ligandation leads to a low level tonic activation of the canonical survival signaling pathways through MEK-ERK and PI3-kinase-AKT (85), providing for possible survival signaling. We have reported that such mechanisms provide chemoresistance in breast and prostate carcinoma (68). This protection functions not only in vitro but also in vivo wherein inhibitors that are not individually effective in limiting or killing metastases re-sensitize the E-cadherin-positive dormant micrometastases to killing by chemotherapies (86). Thus, the disruption of either of these

survival signal cascades brings the response of the dormant metastases to be similar to that of the mesenchymal aggressive and chemosensitive primary metastases.

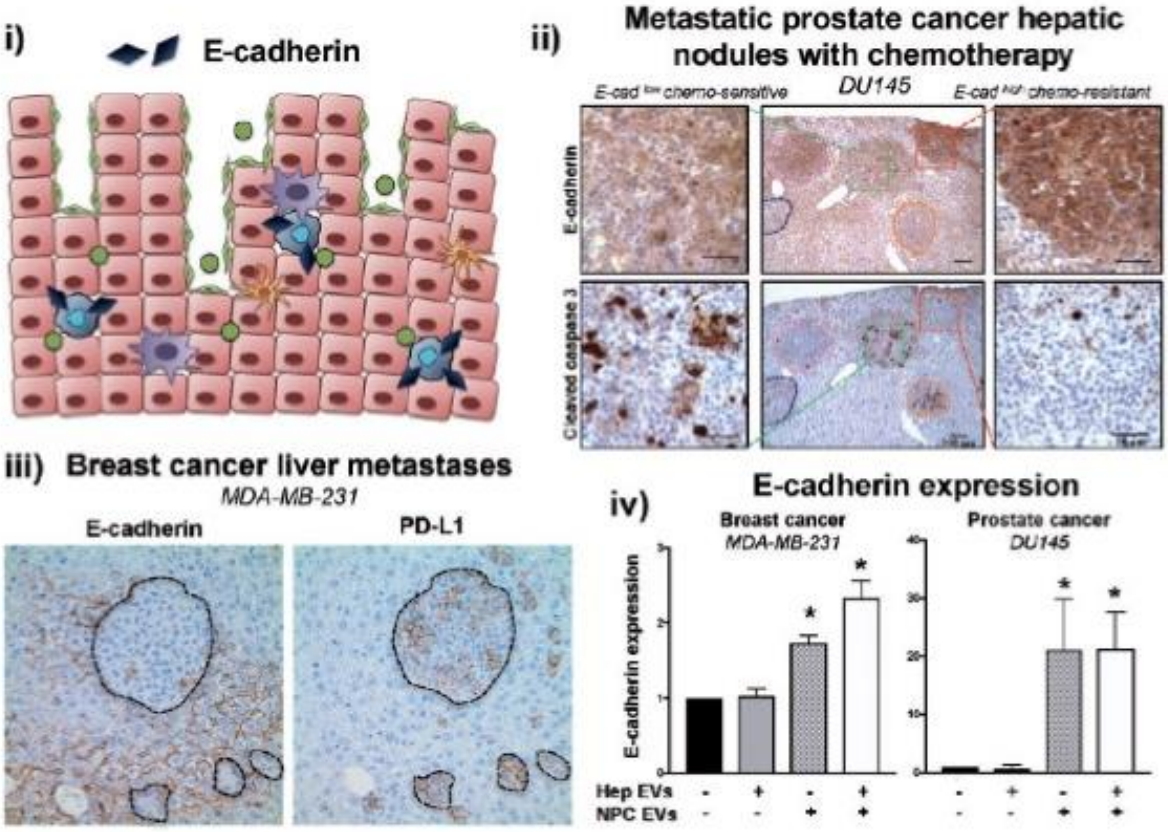

Figure 33: Mechanisms of therapeutic resistance in the micrometastases
Micrometastases escape therapy by up-regulation of survival and down-regulation of target molecules. ii) Tumor cells expressing high levels of the E-cadherin in the liver are more resistant to chemotherapy than those expressing low levels (adapted from (86)). iii) An inverse relationship between E-cadherin (chemoresistant) and PD-L1 proteins (here shown in experimental metastasis models in the mouse, unpublished data) would obviate immunotherapy targeting. iv) EVs from the organ tissue of the metastatic site, particularly the non-parenchymal immune and stromal cells, drive the conversion cMErT in colonizing disseminated cells (adapted from (34)).

Appendix A.10 Resistance of the macrometasteses

The resistance of dormant micrometastases would be less problematic if the emergent macrometastases were responsive to therapeutic agents. As the lethal outgrowths present a mesenchymal phenotype, having undergone a second cEMT, often re-expressing PD-L1, these should be responsive to chemo- and immuno-therapy. Clinical experience suggests that such outgrowths may be in part responsive, as often these shrink or even disappear upon exposure to various chemo-, biologically-targeted- and immuno-therapies (Appendix A - Figure 4). However, these systemic treatments are usually not curative. The almost inevitable recurrence may come from cryptic dormant micrometastases that have avoided therapeutic effects, or from a subset of emergent mesenchymal cells that are protected from killing. If the former, then one would expect the recurrences to display the same sensitivities as the original metastatic growths, whereas if these recurrences derive from surviving cells, a pan- or acquired- resistance should be noted. The localization of the recurrences would not shed light on either of the mechanisms, as even outgrowing macrometastases may harbor dormant nests, and emergent metastases may disseminate and seed secondary sites without undergoing dormancy (4, 87). Clinically, both situations are seen upon treating the recurrences, but most often the resistance profiles are different than the original metastasis (88), suggesting that the latter situation of an adaptive or selected population persisting.

The growing metastasis appears as undifferentiated and mesenchymal, with reduced to absent E-cadherin (7). Such cycling and metabolically active cells should be as susceptible to therapies as the primary lesion; however, this is not the case, and not simply due to exposure-related selection or mutation. The reason for this relative resistance may be that trophic factors in

the metastatic niche are providing survival signals. A candidate for this would be the matricellular protein tenascin C which is present throughout the metastatic bed of melanomas (Figure 11) and

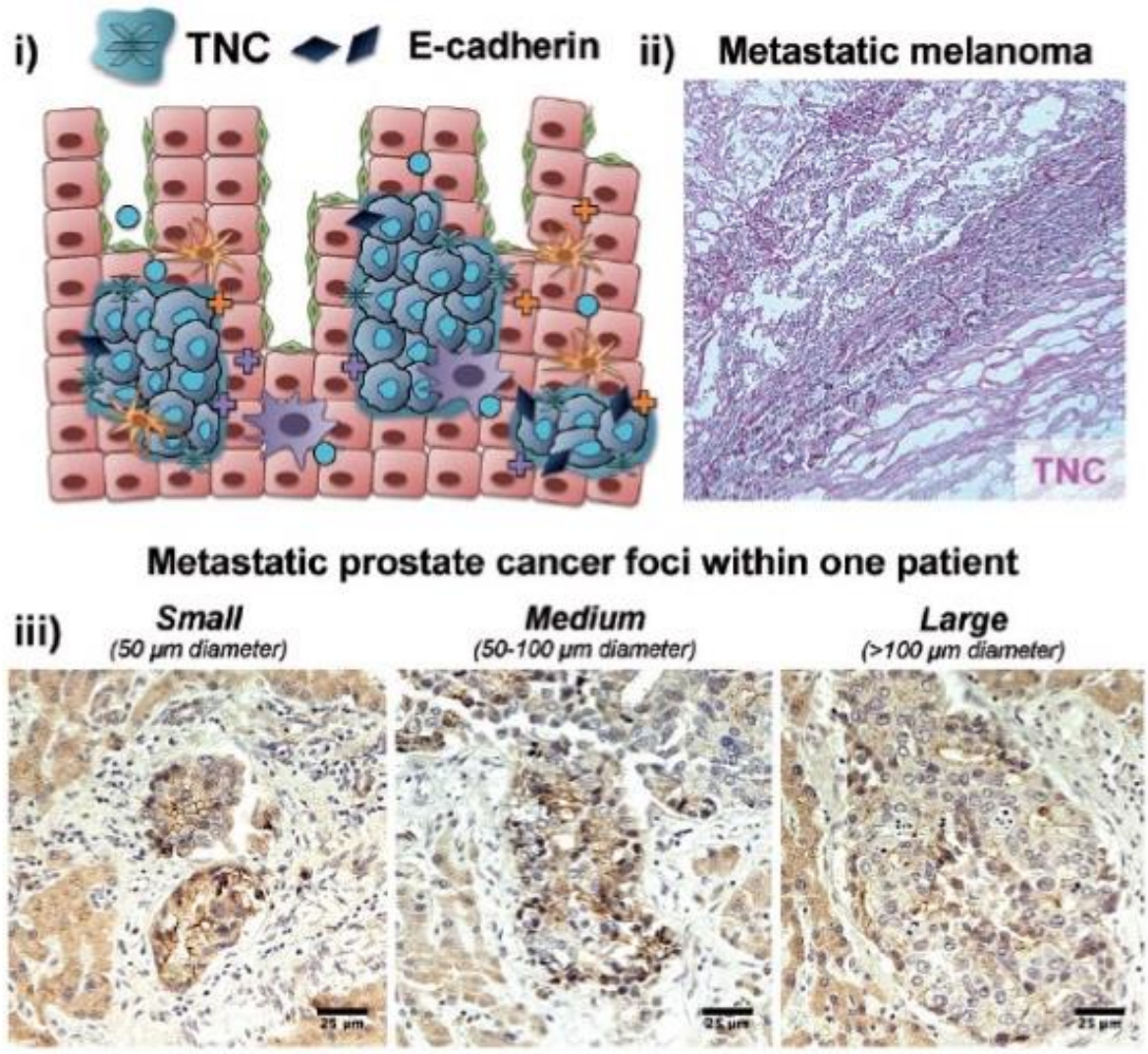

Figure 34: Mechanisms of therapeutic resistance in the macrometastases
i) Mechanisms of therapeutic resistance persists through a second cEMT and subsequent overt growth. ii) Therapeutic resistance of tumor cells is maintained via the production of the same matricellular and matrix-associated proteins (*e.g.*, TNC) produced during the first cEMT event for invasion with this now encasing the entire metastasis. iii) E-cadherin expression inversely correlates with tumor size, with the loss of E-cadherin tracking with emergence as a clinically evident metastatic nodule, opening the way for greater therapeutic sensitivity (adapted from (7)).

other carcinomas (11, 54). Again here, the ultra-low affinity/high avidity EGF-like repeat interaction with the EGFR on the melanoma cell would provide for survival signals. This should be more effective than during initial invasion as the metastases appear to be encased in such a matrix.

Appendix A.11 Commentary

Metastases challenge our ability to cure or ameliorate the cancer due not only to the geographically widespread and often inaccessible nature, but also because they are inherently more resistant to systemic treatments than the primary growth. This escape from elimination is accomplished mainly through non-mutational events based on the plasticity of the tumor cells. The changes in cellular behaviors and phenotypes, shorthanded as epithelial or mesenchymal based on shape and E-cadherin cell surface presentation or lack thereof, respectively, are imparted in large part by the dynamic micro-environments that the melanoma or carcinoma cells progress through. As part of these switches, the successful cells are provided with attributes or signals that provide protection from endogenous killing by the innate inflammation of tumor progression or exogenous toxins of therapies. The survival advantage is greatest in the dormant micrometastases that are not only clinically invisible but avoid detection by the immune system, and whose linked quiescence and E-cadherin-triggered survival signals protect against chemo- and targeted-therapies. Protection persists even after active emergence and growth, though to a seemingly lesser extent, again due to changes in the matrix of the metastases, including expression of matricellular protein tenascin C.

This model accounts for the changes in sensitivity to treatments through progression. However, many of these steps have not been demonstrated in vivo. The main issue is whether macrometastases recur after treatment due to encompassed dormant nests or from adaptation of a portion of the macrometastases. The basis has immediate implications for developing new approaches to treat these resistant growths. If it is from adaptive changes, then this needs to be accounted, versus the outgrowth of quiescent dormant cells, in which case the challenge is to prevent these cells from being awakened, or resensitizing the dormant cells. If this can be discerned, then we can prevent tumor cells from escaping treatment.

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Appendix B - Mesenchymal stem cell/multipotent stromal cell augmentation of wound healing: lessons from the physiology of matrix and hypoxia support.

Text from this chapter appears in *American Journal of Pathology* as a review on stem cells and wound repair.

Synopsis

Cutaneous wounds requiring tissue replacement are often challenging to treat and result in substantial economic burden. Many of the challenges inherent to therapy mediated healing are due to comorbidities of disease and aging that render many wounds as chronic or non-healing. Repeated failure to resolve chronic wounds compromises the reserve or functioning of localized reparative cells, particularly in wounds that require new tissue formation. Transplantation of mesenchymal stem cells (MSCs) have been proposed to augment the reparative capacity of resident cells within the wound bed to help overcome complications in wound healing. However, MSCs face a variety of challenges within the wound microenvironment that hinder their survival post transplantation. Ischemia is considered one of the largest hindrances within the chronic wound microenvironment and has become a major focus for MSC therapies. MSCs are naturally pro-angiogenic and numerous techniques have been attempted to improve their survival and efficacy post transplantation; many with little impact. These setbacks have prompted researchers to re-examine the normal wound bed physiology, which have resulted in new MSC transplantation methods using extracellular matrix (ECM) proteins and hypoxia preconditioning. These studies have also led to new insight on associated intracellular mechanisms such as autophagy which plays a key role in further regulating MSC survival and paracrine signaling. This review provides a brief overview of cutaneous wound healing with discussion on how ECM proteins and hypoxia can be utilized to improve MSC retention and therapeutic output.

Appendix B.1 Introduction

The skin is one of the largest organs of the body and plays an integral role in maintaining homeostasis with the world around us through three critical functions: regulation, protection, and sensation. Upon damage to the skin we can lose one or more of these functions and subsequently kick start the cutaneous wound healing machinery to restore balance. In most cases for patients who are young and healthy, there will be a successful wound resolution leading to restoration of normal skin functions. However, patients who are older or suffer from an array of diseases such as cardiovascular diseases, diabetes, or obesity have significantly higher impairment in their wound healing machinery. Currently in the United States 8.2 million Medicare beneficiaries require interventional wound treatments costing roughly $100 billion dollars[1]. The number of these patients is expected to increase over the next decade due to increasing age of the population compounded by the rise in comorbidities of chronic diseases.

These challenges compromise the reserve or functioning of localized reparative cells, particularly in wounds that require new tissue formation. Mesenchymal stem cells, or multipotent stromal cells (MSCs), have been proposed as a treatment to augment the reparative capacity in patients needing a biological boost to their non-healing wounds. MSCs have become coveted for their pro-angiogenic and immune-modulatory properties in the regenerative medicine space. However, one major complication limiting the efficacy of MSCs are their poor survival rate post transplantation. This is due to the hostility of the wound microenvironment with ischemia being one of the most frequent challenges to overcome. This has led to the development of various strategies to improve MSC survival and angiogenic efficacy to overcome the ischemic barrier. While many of such approaches are pharmaceutical or genetic modifications to the MSCs themselves, several research groups have attempted to harness the physiological survival and

promotion signals native to a healthy wound healing microenvironment. Herein, we will focus on MSC combinational strategies utilizing matrix proteins and hypoxia as natural influences from the wound bed to augment survival and efficacy when used as exogenous cells to promote near regenerative cutaneous wound healing.

Appendix B.2 Cutaneous Wound Healing

Surface wounds are broken down into two major categories depending on the need for tissue regeneration during healing. Surgical wounds and simple cuts are restored by juxtaposing the open wounds edges together and closed mainly by localized matrix remodeling. However, when wounds, such as burns, abrasions and traumatic wounds present with lost tissue, a complex cascade of events ensue (**Figure 32**). An initial hemostatic and inflammatory phase limits the exposure to the external environment and restores a sterile barrier. This is followed through a tissue replacement phase and to successful resolution (scar). However, if complications occur during the early stages of healing with setbacks such as an insufficient immune response or hindered angiogenesis, then the wound will often become chronic or non-healing. Chronic wounds are defined as ulcers or open wounds that fail to resolve within 3 months and are further classified into three main subgroups: vascular ulcers, pressure ulcers, and diabetic ulcers [2, 3]. The severity of chronic wounds will be unique for each patient and will need to be treated with a multifaceted approach by the clinicians. If unable to achieve wound healing advancement then patients run the risk of infection, further spreading of damaged tissue, and amputation. Due to the gravity of repercussions non-healing wounds present, this is the area where scientists and clinicians are most

focused on implementing new biological therapies. In contrast to chronic wounds, abnormalities in the last two stages of wound healing can result in a wound with excessive scarring such as keloid or hypertrophic scar[3,4, 5]. While the wound appears epithelialized and 'healed', these scars are dysfunctional and have an underlying dermis that is disfigured and compromised. The improper healing in these scars result in wounds that close but are structurally weaker and prone to re-ulceration.

The complete wound healing cascade is triggered when an injury damages the dermal and subdermal layers of the skin. The subsequent wound healing response is an intricate orchestration of three overlapping phases of repair that encompass numerous cell types, signaling cascades, and microenvironment modifications to reach a successful resolution (**Figure 32**). There are three main phases of wound healing: the hemostasis / inflammation phase, the tissue replacement phase, and the resolution phase. The first phase of wound repair is the hemostasis / inflammation phase where first point of concern is to stop the bleeding of ruptured blood vessels while subsequently preventing pathogenic infection. Upon injury, a clotting cascade is activated where blood circulating platelets and enzymatic converted fibrin come together to form a fibrin clot and provide an early provisional matrix over the wounded area[6]. This fibrin clot is also biologically active playing a major role in signaling a localized immune response through the recruitment of pro-inflammatory macrophages (M1) and leukocytes to prevent infection and clear the wound of cellular and extracellular debris.

The next phase of wound healing, the tissue replacement phase, begins with the in-migration of fibroblasts, endothelial cells, epidermal cells, and other progenitor cells into the

wound bed to initiate the rebuilding of the injured tissue. Fibroblasts begin producing the collagen 3-rich granulation tissue as a temporary supportive matrix to replace the absent extracellular matrix

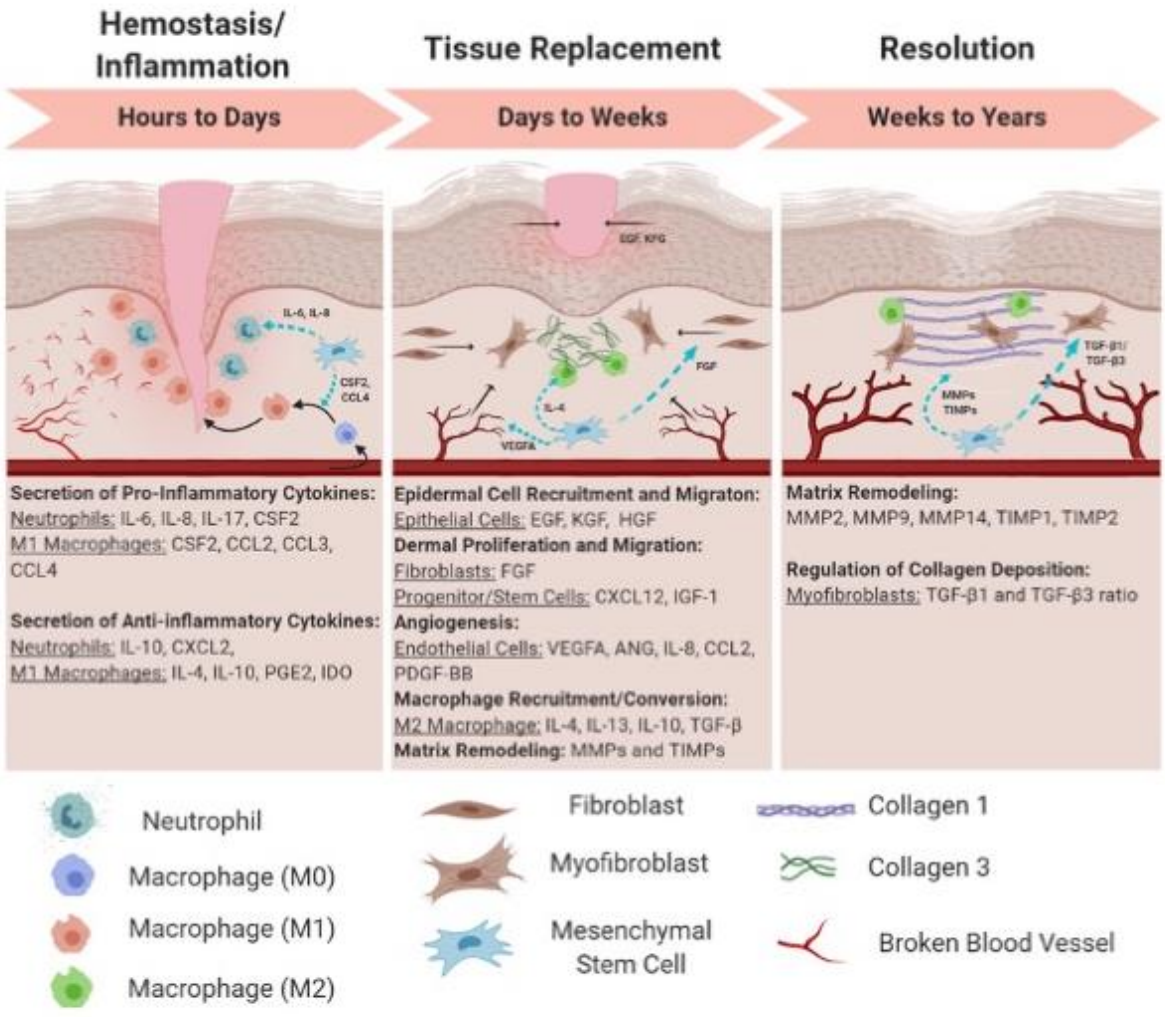

Figure 35: Roles for MSCs during cutaneous wound healing
The hemostasis/inflammation phase is heavily reliant upon the pro-inflammatory machinery for sterilizing the wound and clearing damaged ECM debris. MSCs help to orchestrate a healthy initial immune response that eventually will turn over into a pro-reparative response. Upon entering the tissue replacement phase, MSCs produce various growth factors and chemokines to initiate a massive arrival of tissue resident cell types that will begin to rebuild within the wound bed. The resolution phase is the final phase of wound healing where MSCs help to orchestrate final modifications to the extracellular matrix, vasculature, and resident cells.

(ECM); endothelial cells begin to create new blood vessels via angiogenesis; and the epidermal cells start to migrate underneath the scab to permanently seal the wound surface and restore the epidermis. At this point the wound is considered sterile and a transition occurs where pro-inflammatory macrophages (M1) decline and are replaced by wound healing macrophages (M2). These M2 macrophages help to produce and modulate the granulated ECM and promote further vasculature repair.

The resolution phase is the final repair phase where the provisional wound bed is replaced with a mature ECM, the excess blood vessels are pruned by up to 90%[7], and any residual proliferation and migration signals are terminated and replaced with 'stop' signals such as those acting through CXCR3 [8]. The wound bed will start to contract as fibroblasts transdifferentiate into myofibroblast to reorganize and restructure the immature collagen 3 to mature collagen 1, restoring tensile strength back to the skin[9]. With every phase having a successful outcome, all that will remain will be a light scar to hint at what occurred.

Appendix B.3 Mesenchymal Stem Cells in Wound Repair

Mesenchymal stem cells, or multipotent stromal cells (MSCs), are characterized for their ability to self-renew, adhere to plastic, and differentiate into subsets of specialized cells particularly suited for the regeneration of mesenchymal tissues (adipocytes, osteoblasts, chondrocytes, and myoblasts)[10-16]. They are further defined by the International Society for Cell & Gene Therapy for expressing markers: CD73, CD90, and CD105; while also lacking the expression of markers CD14, CD19, CD34, CD45, CD11b, CD79a, and HLA-DR[17]. MSCs as a

cellular therapy has shown clinical benefit in several disease situations, though this has related to the immunosuppressive activities rather than tissue generation[18, 19]. Unfortunately, this approach has proven disappointing in wound repair despite success in pre-clinical animal models as the stem cells are lost rapidly from the wound bed. A more recent area of success using MSCs has been for angiogenesis and improved healing amongst different tissue types. This is due to the variety of trophic factors that MSCs have been found to secrete in context to their microenvironment[18, 19]. Still, to achieve any significant benefit, the MSCs must remain for the time scale of the wound healing phase, which run in the weeks.

The use of MSCs to treat dysfunctional cutaneous wounds is considered particularly promising for a number of reasons. First, the immune-tolerant site of the skin can accommodate extended persistence of allogeneic cells. Second, skin being an externally accessible organ, repeated applications can be achieved non-invasively. In addition, the pro-reparative secretome of MSCs can be utilized in different phases of the wound healing process **(Appendix B – Figure 32).** This is an advantage over direct application of growth factors or other molecules as the wound bed consists of asynchronous areas of healing; where MSCs are environmentally adaptive and can produce an appropriate response for the transitioning phases of the wound bed to recreate the physiological situation. Such as in a normal skin wound, where endogenous subsets of hair follicle MSCs called dermal sheath cells help to repair/replace the injured dermal tissue. While MSCs from the subcutaneous fat tissue and blood supply help regulate early and mid-phase inflammation while also helping to restore dermal tissue[20, 21].

Upon infiltrating the wound, MSCs begin to secrete pro-inflammatory cytokines (CSF2, IL-6, IL-8, CCL2, CCL3) recruiting neutrophils and M1 converted macrophages to help degrade damaged tissue[21, 22] (in the Hemostasis/Inflammation phase), but also regulating the overall

inflammatory response by limiting the number of activated T-cells, neutrophils, and macrophages[21, 23-25]. Upon transition into the Tissue Replacement phase, MSCs focus on secreting cytokines directed to the proliferation and migration of epithelial cells for re-epithelialization (EGF, KGF, HGF), directing new endothelial cells for angiogenesis (VEGFA, ANGPT1, PDGF-BB), and stimulating fibroblasts to produced matrix proteins that later transition to a mature provisional matrix (FGF)[21, 22, 26, 27]. MSCs also direct the polarization of monocytes into pro-reparative M2 macrophages (IL-4, IL-13, IL-10, TGF-B) to help clear remaining cellular debris and modify temporary matrix[21, 24]. In the Resolution phase, MSCs continue to regulate the matrix through secretion of MMPs and TIMPs, while also regulating the amount of collagen disposition through balancing TGF-B1 and TGF-B3 to prevent hypertrophic scarring[27]. These vital guiding functions are unique to MSCs because they express low levels of major histocompatibility complex class (MHC) II along and lack the MHC co-stimulatory molecules which are essential for immune cell activation (CD40, CD40L, CD80, CD86); allowing for their use in allogeneic transplant with a very low rate of rejection[28].

Appendix B.4 Hostile Wound Microenvironment Challenges MSC Survival

Despite the potential benefits MSCs possess for regenerative therapies, their use must first overcome pro-apoptotic stressors within wounded tissue[29]. Younger and healthier patients have wound healing machinery and endogenous MSCs that work well enough to overcome these healing challenges. However, in patients who are older and suffer from diseases such as those with type 2 diabetes or metabolic syndrome, their MSCs become more susceptible to apoptosis, increased levels of ROS accumulation, and increased mitochondrial deterioration. Rendering their MSCs too

dysfunctional[30] to be able to overcome the stress of the wound bed ultimately limiting their effectiveness in the wound healing process. This is where the reliance on exogenous MSCs therapies from healthier and younger donors has come to light. However, the lack of survival of exogenously applied MSC in wounds has still been well documented in a variety of tissues, particularly the heart[31], brain[32], and kidney[33]. For the heart, a survival rate of 5% within a two-week period post myocardial infarction (MI) treatment [31], and only a 1% survival rate one hour after injection into an ischemic kidney model [33]. Even when using an immunodeficient mouse model, Toma et al. report having less than 0.44% survival of MSCs 4 days post MI [34]. Even skin, where immune tolerance should present less barriers to MSC, is devoid of implanted MSCs within a week [35].

The disparagingly low survival rates likely result from a variety of factors within the harsh wound microenvironment, such as the absence of trophic factors, a heightened inflammatory response, and an impaired vasculature [36, 37]. These factors induce MSC death through a variety of mechanisms, including anoikis, ischemic reactive oxygen species (ROS), loss of growth factors, or increased signaling from death cytokines[38.](**Figure 33**). Our lab has previously shown that MSCs are very susceptible to ROS and pro-inflammatory death inducing signaling complexes that occur within the wound bed [39]. We have also shown that MSCs are metabolically glycolytic [40], so that nutrient deprivation within the wound bed is also potentially detrimental to MSC survival [41, 42]. Recent evidence regarding hypoxic/ischemic stress showed that severe hypoxia (<1% O_2) drives mitochondrial dysfunction resulting in apoptosis or necrosis [41, 43]. Despite numerous approaches to limit MSC death post transplantation whether through growth factor or drug preconditioning or genetic modifications [44]; most efforts have had a limited impact on overall MSC survival. The death of the exogenously applied MSCs is in counterpoint to the survival of the endogenous MSCs.

Therefore, we can learn from the physiological adaptations and survival mechanisms of successful resolving wounds to develop pro-survival therapies for those who have a dysfunction of their own MSCs, the elderly and those with co-morbidities.

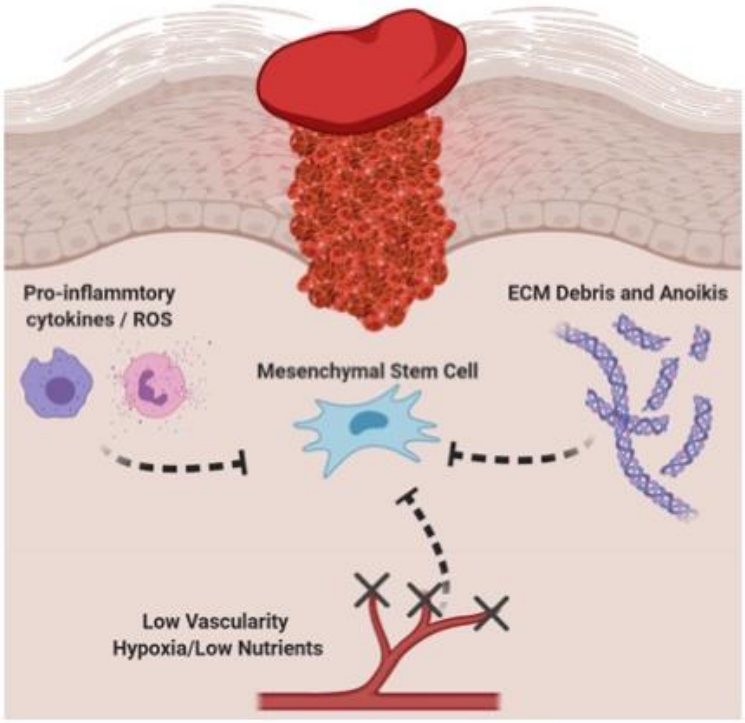

Figure 36 MSCs face a steep challenge in the wound microenvironment
Several factors are in play that will limit their survival and therapeutic capacity. The innate immune response is responsible for destroying any foreign pathogens, but their methods of destruction can be non-discriminatory through their release of pro-inflammatory cytokines and reactive oxygen species (ROS). The insult will also cause tissue damage, leaving behind ECM debris that can result in a lack of support to cells in the area, resulting in cell death via anoikis. Injury to the wound bed can also cause blood vessels to be severed subsequently resulting in all damaged vasculature to be blocked off. This will cause the wound microenvironment to become ischemic leaving all cells to be with little to no oxygen or nutrients available.

Appendix B.5 MSC Delivery Strategies

Cutaneous wounds requiring interventional treatment options are often complex and require a multidisciplinary approach both at the macro level and micro levels of care. Where macro levels of care would be considered more general hospital methods of treatment such as site off-loading, compression bandages, and fluid control. Micro level of care would be anything using a biological treatment focused on improving the wound at the microenvironment level. There are many elements clinicians and scientists must consider when utilizing and developing MSC therapeutic strategies. Cell source is often a major consideration because there are multiple types of MSC derived lineages that can behave differently under varying circumstances. In addition, there are some sources of MSCs that are easier to extract and with greater numbers than with other locations of the body. The two most common sources of MSCs for clinical use are either derived from the bone marrow where 0.001-0.002% of cells harvested are MSCs; or from adipose tissue where MSCs are estimated to make up 1% of the total cell population[45]. Cell administration strategies is another key factor and will change depending on the patients wound severity and type. But overall the main goal of improving MSC survival percentages post transplantation remains a focal point for enhancing MSC efficacy in wound healing. Here we focus on two of the most promising approaches for improving MSC retention: extracellular matrix construct and hypoxic preconditioning. Both of these methods rely on the impact of environmental cues upon the MSCs to influence their behaviors rather than artificial micro manipulations such as gene editing techniques or pharmaceutical drugs. The overarching idea for these MSC delivery strategies are to utilize components native to the wound healing environment / repair mechanisms in order to reduce extraneous stress other more artificial methods may add to an already dysfunctional system. Both extracellular matrix compositions and hypoxia/ischemia are central aspects of the

inflammatory and tissue replacement phases of wound healing, and thus can be considered physiological mimics.

Appendix B.6 Matrix Components for MSC Delivery

The extracellular matrix is now appreciated as being just as important as the cells themselves during healing [3, 40]. The ECM is not just the major support structure of each tissue, but the tension combined with bioactive moieties within the ECM largely influences how cells within the tissue function and behave. The ECM's biochemical makeup, rigidity, and shape are being exploited to manipulate tissue replacement and wound healing across many applications. Using ECM constructs that either resemble or impute the native matrix environment could help in promoting more physiological healing.

Collagen-1 is the most abundant ECM protein of the skin and is usually preferred for shovel-ready scaffolds. Collagen-1 is biocompatible, biodegradable, and can form highly organized 2-D and 3-D network like structures that allows it to incorporate a wide variety of biological components ranging from growth factors to matrix proteins to cells. Collagen-1 has been shown to improve MSC proliferation potential and prevent MSC anoikis through the binding of integrin receptors α2/β1, and α11/β1[46, 47]. Collagen-1 is also able to increase osteogenic differentiation through higher cell adhesion and enhance activation of RHO-A *in vitro* [46]. Another structural collagen, Collagen VI, has been used to enhance MSC proliferation and stemness for cartilage repair, while also displaying pro-survival capabilities in fibroblasts [48, 49]. Thus, encapsulating MSCs within structural collagen may augment the delivery of viable MSCs. However, Collagen-1 signals wound maturation and resolution, and thus would be suppressive

during the early tissue replacement phase of healing; importantly collagen-1-dominant matrices limit angiogenesis [40].

A more immature matrix is likely needed to promote regenerative healing [50]. Previous work in our lab has shown that the matricellular protein Tenascin-C (TNC) possesses epidermal growth factor receptor-like (EGF-L) repeats that can interact with the EGF receptor (EGFR) on MSCs in a low affinity/high avidity interaction; this restricts EGFR activation to the plasma membrane [29,51]. The prolonged sequestration of EGFR enhances pro-survival signals, via low level tonic ERK and AKT signaling [37,52]. The addition of TNC to MSCs provides for survival of the transplanted cells for up to a month [26, 35]. Given that TNC is found naturally during the early phases of wound healing, coupling it with MSCs as a therapeutic delivery system is expected to not only provide for enhanced survival but also improve healing [26, 35]. Laminin V is another promising immature matrix protein that contains cryptic EGFR matrikines[52]. Combinational strategies using Laminin with MSCs have shown to enhance overall survival and improve wound healing outcomes through upregulated angiogenic capacity in diabetic rats and other models[53, 54]. The wound response matrix component fibronectin is another top candidate for MSC delivery. MSCs survival was enhanced in a rat hind limb model when encapsulated in agarose capsules containing fibronectin and fibrinogen pro-survival signals[55], in addition to increasing proangiogenic capacity of MSCs *in vitro*[54].

In addition to improving MSC survival and paracrine signaling, many of these immature matrices also promote the migration of cells around the wound bed. Tenascin-C, laminin, fibronectin, and thrombospondin all play vital roles in orchestrating the movement of distal proliferating resident cells to the center of the wound bed mainly through integrin binding mechanisms[56-58]. These properties add another facet to the overall capability of these MSC-ECM

combinational therapies. Where the immature matrices improve the survival and growth factor secretion of MSCs, which then promotes enhanced proliferation of the resident tissue cells, that in turn are able to migrate into the wound quicker due to the aforementioned present immature matrix. Further adding to this enhanced cycle of repair, MSCs have also shown that they can deposit ECM proteins such as fibronectin, tenascin-C, thrombospondin and others[59]. All together these matricellular proteins are highly regulated in adult tissues with restricted expression to areas of active remodeling, such as in wound healing [60]. Thus, by following the lessons from physiological healing, novel approaches can be designed employing the same matricellular proteins.

Appendix B.7 Hypoxic Preconditioning for MSC Delivery

Many MSCs reside in areas of low oxygen-tension from 3-9% depending on the tissue source [61, 62]. Hypoxia within the stem cell niche is thought to maintain self-renewal, proliferation, migration, and ultimately appears to augment their therapeutic potential [63-65]. Taking these cues from physiology, this has led to experiments in which MSCs are grown at similarly low levels of oxygen to condition or acclimate them prior to wound implantation. However, when oxygen levels reach extreme levels of hypoxia (<1.5% O_2) such as within a wound bed, MSCs can become over stressed and undergo apoptosis [64]. Further studies have shown that MSCs are innately glycolytic [40], and it is the combination of low oxygen tension and nutrient deprivation within the ischemic wound environment that severely challenges the cells. MSCs exposed to near-anoxic conditions (0.1% O_2) for as little as three days were shown to rapidly consume all internal glucose energy reserves resulting in poor survival post implantation [66].

With both oxygen and nutrients being vital for MSC survival post implantation, researchers are focused on creating delivery methods that improves at least one of these bioenergetic challenges. The biggest and potentially easiest way to enhance MSC survival is to pre-condition MSCs in hypoxic conditions (1-4% O_2) for 24 to 48 hours prior to implantation. MSCs pre-exposed to these hypoxic conditions respond by upregulating the hypoxia inducible factor 1α (HIF-1α) [67]. HIF-1α is a major regulator and response factor to oxygen tension within the cell, in which it will be post-translationally modified and marked for degradation under normal oxygen conditions. During hypoxia, HIF-1α escapes degradation and translocates to the nucleus where it will then activate hypoxia response elements (HREs). Many of the genes that HREs target are involved in promoting angiogenesis (i.e. Vascular endothelial growth factor, VEGF), survival (Bcl-2, AKT), or regulate metabolism via increasing glycolytic potential (i.e. glucose transporter 1 - GLUT1, lactate dehydrogenase A – LDHA, phosphoglycerate kinase 1- PGK1) [68, 69]. The increase in glycolytic genes translates to increased MSC consumption of glucose and production of lactate in an oxygen-dependent manner [64, 70]. Further analysis of HRE targets genes have also found active mitochondrial repressors such as pyruvate dehydrogenase kinase 1 (PDK1); an inhibitor of pyruvate entrance into the TCA cycle. PDK1 inhibition of pyruvate effectively reduces mitochondrial oxygen consumption and thus allows cells to preserve their intracellular oxygen concentrations [71]. MSCs exposed to hypoxic conditions also utilize HIF-1α to activate the AKT signaling pathway to enhance survival and proliferation [72]. However, when HIF-1α is knocked-down, compensatory activation of the p53 pathway occurs resulting in a higher induction of apoptosis [69]. These manipulations appear to marginally improve the survival of the transplanted MSCs[70, 73].Thus, the next steps would be a combination of hypoxic preconditioning with delivery within matricellular components such as TNC.

Appendix B.8 Autophagy May Be Key Mechanism for Survival and Efficacy in MSC Combinatorial Therapy

Upon further investigation into the effects of hypoxic preconditioning on MSCs, upregulated HIF-1α was shown to promote the initiation of macroautophagy [74]. Macroautophagy (hereafter, autophagy), is an evolutionarily conserved "self-eating" catabolic process that targets cellular components for degradation through the formation of double membraned organelles called autophagosomes that later fuse with lysosomes [75]. Under normal circumstances, autophagy is a highly selective process used to maintain cellular homeostasis by degrading the buildup of aggregate-prone proteins and dysfunctional organelles [76]. However, when cells are exposed to environmental stressors such as nutrient deprivation or hypoxia, autophagy becomes a non-selective process indiscriminately sequestering large amounts of cytosolic cargo for degradation in the attempt to prolong survival [77]. The activation of non-selective autophagy quickly becomes a balancing act within a cell, as autophagy can play a role in both cell survival and apoptosis [78]. In addition, autophagy and apoptosis mutually inhibit each other until an undefined sensitivity threshold is reached and one process overtakes the other; subsequently resulting in either adaptation of the cell to the stress or stressed induced death [43, 79]. It is through these threshold boundaries where MSCs can be further fine-tuned to escalate their efficacy as cell therapies.

MSC performance and function are tied to its ability to regulate autophagy[80]. To date, basal autophagy levels have been shown to be higher in MSC populations compared to differentiated cell types [81]. Additional studies suggest that autophagy is required for conservation of MSC stemness and self-renewal capabilities [82]. Even more interesting is that non-stressed MSCs accumulate arrested autophagosome, and their autophagic manipulation significantly alter the balance between renewal and differentiation [83]. MSC directed differentiation dynamics will change

when autophagy is manipulated as early as 3 hours after induction into adipogenic and osteogenic lineages [83, 84]. This is due to the MSCs quickly consuming the arrested autophagosomes that were present prior to induction, and by speeding up or slowing down the autophagy process, this will either increase or decrease the overall differentiation efficiency outcomes [83].

MSC paracrine function has also been linked to autophagic manipulation. This has been reported mostly in conjunction with hypoxic preconditioning. As MSCs experience hypoxia, HIF-1α becomes upregulated and allowed to translocate into the nucleus, it initiates the production of BNIP3/BNIP3L, a set of pro-autophagy proteins that interact with the Bcl-2 sequestration complex allowing Beclin-1 to dissociate away and initiate the autophagic cascade [85]. In addition to BNIP3, hypoxia also positively influences autophagy induction through regulating the expression of MAP1LC3β and ATG5 via PERK-UPR pathway, as well as activating the AMPK pathway [86]. This upregulation in autophagic flux has been linked to increased expression of VEGF secretion through direct phosphorylation of ERK [87].The combination of pretreating MSCs with hypoxia could enhance the therapeutic potential of MSCs even further especially by promoting angiogenesis [88]. In treating post MI hearts, MSCs that underwent hypoxic preconditioning exhibited higher rates of autophagic flux resulting in higher retention in cell number and a significant reduction in scar formation within the infarct wall [89].

The strong connection of autophagy to cellular processes such as angiogenesis has launched the cancer treatment and stem cell therapy fields to find additional upstream activation/regulation inputs. This has resulted in a large emphasis on the ECM as a point of interest for such studies[90]. The most recent studies have focused on proteoglycans, primarily the small leucine rich proteoglycan called Decorin[91, 92]. Decorin becomes upregulated within the resolution phase of wound healing where mature matrix such as Collagen-1 becomes present again during

this final stage of healing. Decorin is considered an important shut off switch for the pro-reparative mechanism of the tissue replacement phase, as it binds to growth factor receptors such as EGFR, VEGFR2, and MET; and limits the occurrence of hypertrophic scarring through preventing excessive repair. Upon binding these receptors, Decorin will induce autophagy and mitophagy responses within the cell[91, 93]. However, these actions have been shown in differentiated cells and not yet in stem cells. It is also unclear of the physiological roles Decorin induced autophagy has in certain cell types[91]. When postulating what this could mean for MSCs, Decorin could be helping to reset the stem cell niche back to its dormant state by downregulating the proliferation and angiogenic signals once needed for early wound repair. And like other cells that remain in a quiescent state for long periods of time, they rely heavily on autophagy to clear out unwanted intracellular debris unlike higher proliferating cell types.

For early wound healing situations, The immature matricellular TNC has been linked to prompt Beclin-1 induction[94] which could possibly induce autophagic flux. This would either be a second mode of survival mechanism (in addition to the tonic AKT and ERK signaling) or an additional driver in producing the secretome for tissue replacement. TNC is also known as being an anti-adhesive matrikine, allowing for the migration and ECM detachment of cells during development and wound repair[94]. Loss of ECM attachment has been shown to induce autophagy to promote a survival mechanism against short term anoikis[95]. Thus, the anti-adhesive nature of TNC along with other ECM anti-adhesive glycoproteins could render a secondary method of action for inducing autophagy survival mechanism in MSC-ECM transplantation strategies. In order for

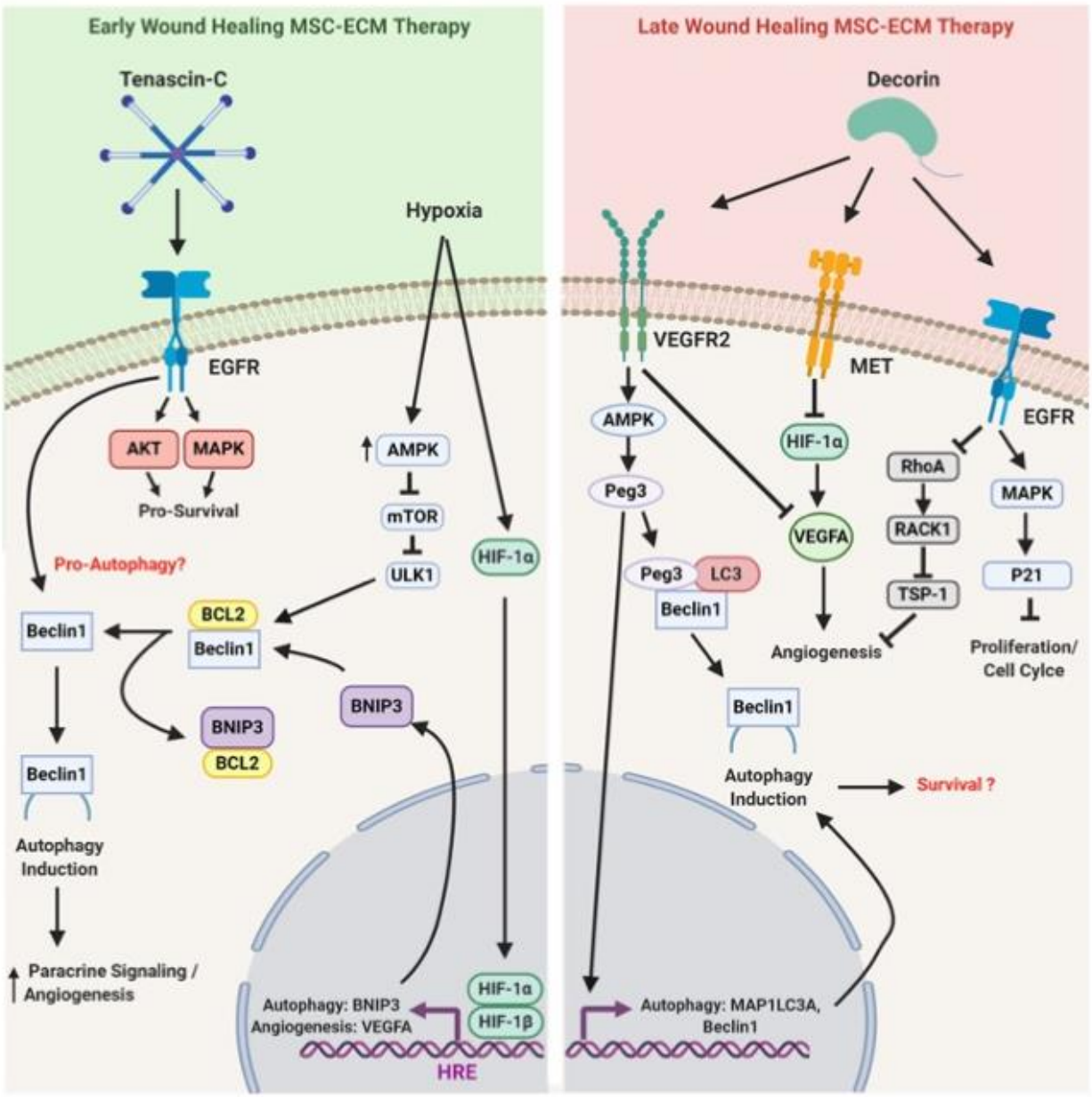

Figure 37: MSC-ECM combinational strategies need to be customized

Depending on the target stage of wound healing MSC will have different intracellular influences on performance. The left panel represents an MSC-ECM combinational therapy targeting the inflammatory and tissue replacement phases of wound healing such as in treating a chronic ulcer. Using a matrix protein that is naturally occurring during the early phases of wound repair, TNC will provide pro-survival signals through continual ERK and AKT signaling cascades. In conjunction with the TNC survival signals, the MSCs will be exposed to hypoxia/ischemia. This will jump start the HIF-1α signaling complex and result in an upregulated autophagic (BNIP3) and pro-angiogenic response (Hypoxia Response Elements such as VEGFA). TNC is also linked to the induction of Beclin1 and has been postulated as an influencer of autophagic expression. With the addition of the autophagic machinery the MSCs can utilize its output of nutrients / building blocks (amino acids, nucleic acids, fats, sugars) to further enhance its pro-angiogenic

(Continued) signaling properties. In the right panel we have an MSC-ECM combinational therapy that would be used to target the resolution phase of wound healing in hopes of stopping excess scar formation. Decorin is a naturally occurring matrix protein during the resolution phase and works as a major stop signal for all the pro-wound healing systems that were at play in the early phases of repair. It binds to various growth factors and receptors where it signals the surrounding cells to stop proliferating (EGF), and stop producing angiogenic factors (VEGFR2, MET, EGFR). However, Decorin is well known for inducing a robust induction of autophagy (VEGFR2) without a real clear understanding of how that effects the physiology of the cells moving forward.

majority of these anti-adhesive ECM proteins to be properly combined with MSC delivery strategies in 2D or 3D, Col-1 is often used has a structural tether to keep everything together. However, there is evidence *in vitro* that Col-1 actively down regulates autophagy[96], suggesting that further research will be needed to fine tune the ratio of matrix protein delivery strategies depending on the required outcome. This concept is even more important when considering what phase of wound healing is needing to be targeted. Where a pro-survival ECM protein like TNC is useful during the inflammation and tissue replacement phases of repair to help restore delayed wound healing; and Decorin is most useful during the resolution phase of wound healing to stop the pro-reparative system from over producing and creating unwanted scarring (**Figure 34**). Altogether, both environmental stimuli of hypoxic preconditioning and matrix proteins are linked to autophagy in various ways, and further investigation into how to finetune their inputs in conjunction with autophagic flux could prove to be a key insight into optimizing MSC therapeutic approaches. Especially in essential processes such as angiogenesis, where increased autophagic flux has been linked to upregulating angiogenesis through increased production of VEGFA in cutaneous wound repair[87].

Appendix B.9 Conclusion

MSCs have numerous attributes that make them attractive for facilitating healing in dysfunctional and nonfunctional wound beds. Their use as adaptive paracrine and matrix producing 'factories' is desirable in that eventually rejected allogeneic cells could still promote near-regenerative healing. However, MSCs face many challenges post transplantation into the wound bed. Overcoming these challenges is necessary to demonstrate their efficacy in treating injured tissue (**Figure 35**). We propose that successful use of MSCs in promoting more regenerative healing or completing stalled chronic wounds will ensue from learning from the physiology. Using preconditioning techniques and matrix support strategies to target autophagic responses could be a key in improving MSC survival[80]. MSCs are inherently glycolytic[40] and will rapidly use internal energy reserves [66] when subjected to extreme environments such as ischemia in wound bed. Manipulating how MSCs utilize autophagic flux could help maintain internal energy stores for longer period of time, leading to larger cell retention. Matricellular component-containing delivery vehicles not only impact autophagy and metabolic programming but also directly trigger survival pathways, further improving the persistence of transplanted MSCs. This increase in survival fraction would allow for the MSCs to exert trophic influences on angiogenesis and tissue replacement for the weeks-long period needed to transition through the phases to wound resolution.

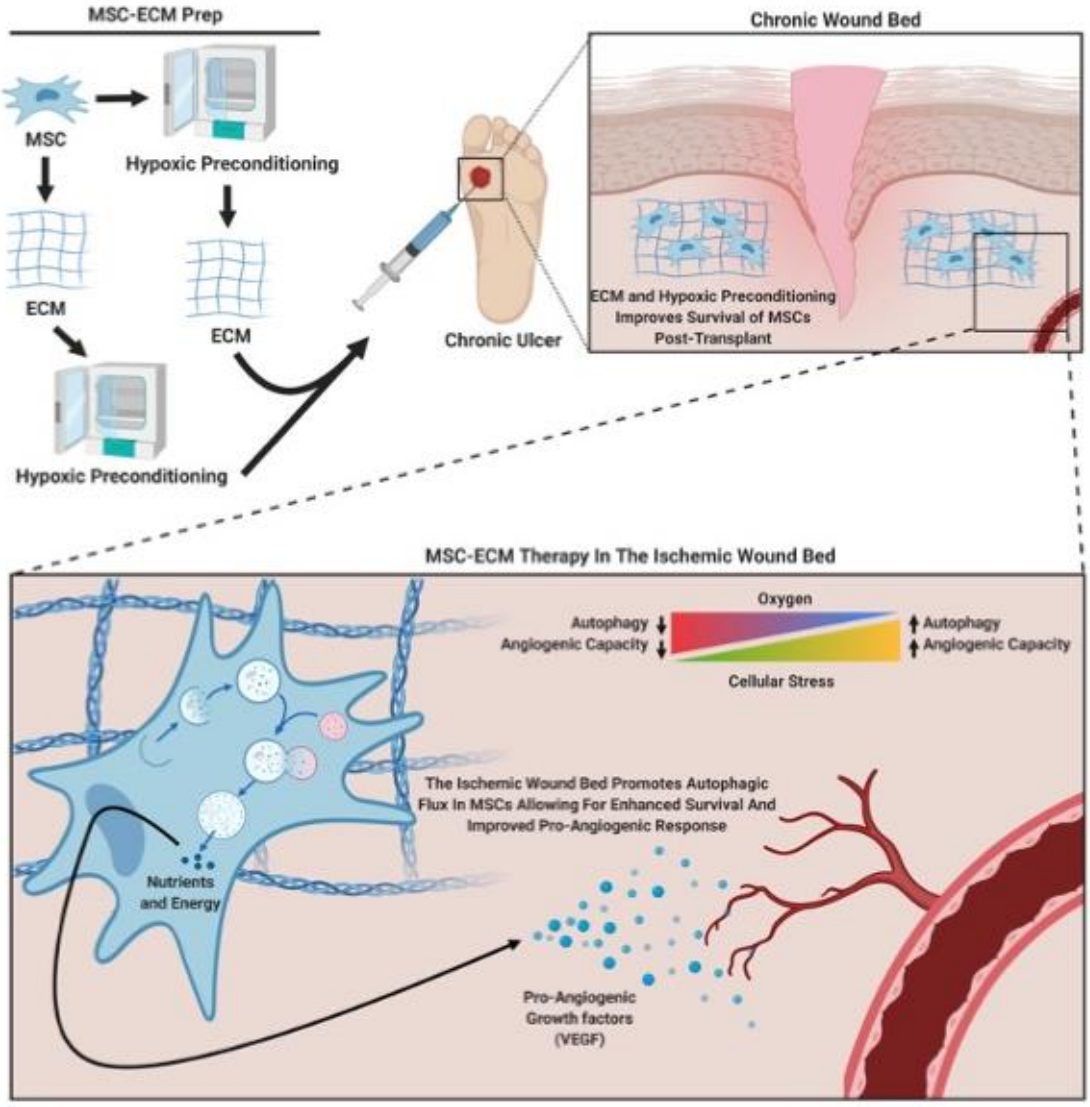

Figure 38: Overview schematic of combinational MSC therapies

First step in the process is to prepare the ECM constructs and MSCs for delivery. The type of skin wound, and the build of the ECM scaffold will determine whether MSCs need to be combined with the ECM before or after hypoxic preconditioning takes place. Once the MSC-ECM sample has been prepped, they can be delivered to the wound. The combination of the ECM construct support and the hypoxic preconditioning will help the MSCs overcome the initial shock of the ischemic wound bed, allowing them to acclimate better to newly encountered cellular stressors. The ischemic environment will promote additional intracellular survival machinery such as autophagy, allowing the MSCs to gain additional nutrients and energy for longer survival. Once the MSCs have stabilized, they will start to respond to the ischemic environment by utilizing the autophagy machinery and produce pro-angiogenic growth factors such as VEGF to promote angiogenesis. The restoration of new vessels will help enrich the surrounding resident cells and ultimately progress the wound forward in the healing process.

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Appendix C – Antibodies

Table 4: Antibodies

Antibody	SOURCE	PRODUCT #	IDENTIFIER
Collagen I	Abcam	ab34710	RRID:AB_731684
MITF antibody [D5]	Abcam	ab3201	RRID:AB_303601
Tenascin C antibody [EPR4219]	Abcam	ab108930	RRID:AB_10865908
E-Cadherin (24E10)	Cell Signaling Technology	3195	RRID:AB_2291471
GAPDH (D16H11)	Cell Signaling Technology	5174	RRID:AB_10622025
phospho-AKT (Ser473)	Cell Signaling Technology	12694	RRID:AB_2797994
pan-Akt (11E7)	Cell Signaling Technology	4685	RRID:AB_2225340
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	Cell Signaling Technology	4370	RRID:AB_2315112
p44/42 MAPK (Erk1/2)	Cell Signaling Technology	9102	RRID:AB_330744
α-Tubulin Antibody	Cell Signaling Technology	2144	RRID:AB_2210548
α-Tubulin (DM1A)	Cell Signaling Technology	3873	RRID:AB_1904178
Decorin	R&D Systems	AF143	RRID:AB_354790
Tenascin-C Antibody (BC-24)	Santa Cruz Biotechnology	sc59884	RRID:AB_785991
α-Smooth Muscle, Anti-Actin	Sigma-Aldrich	A5228	RRID: AB_262054

Appendix D - Chemicals & Reagents

Table 5: Chemicals and Reagents

CHEMICALS	SOURCE	PRODUCT #
Vemurafenib (PLX4032)	Selleck Chemicals	CAT# S1276
Selumetinib (AZD6244)	Selleck Chemicals	CAT# S1008
Vactosertib (TEW-7197)	Selleck Chemicals	CAT# S7530
Infigratinib (BGJ-398)	Selleck Chemicals	CAT# S2183
PD153035 (EGFRi)	Selleck Chemicals	CAT# S1079
SB431542 (TGFβRi)	Selleck Chemicals	CAT# S1067
Alexa Fluor 488 Phalloidin	Thermo Scientific	CAT# A12379
Alexa Fluor 594 Phalloidin	Thermo Scientific	CAT# A12381
Collagen I - High Concentration Rat Tail	Corning	CAT# 345249
Goat Anti-Rabbit IgG Antibody, HRP-conjugate	Sigma Aldrich	CAT# 12-348
Goat Anti-Mouse IgG Antibody, HRP-conjugate	Sigma Aldrich	CAT# 12-349

REAGENTS	SOURCE	PRODUCT #
Control Lenti particles - GFP	Origene	CAT# TR30021V
Control Lenti Particles - RFP	Origene	CAT# TR30033V

Appendix E – Primers

Table 6: Primers

GENE	PrimerBank or REF ID		Sequence (5' -> 3')
TGFβ1 (version 1)	260655621c1	Forward	GGCCAGATCCTGTCCAAGC
		Reverse	GTGGGTTTCCACCATTAGCAC
TGFβ1 (version 2)	260655621c2	Forward	CTAATGGTGGAAACCCACAACG
		Reverse	TATCGCCAGGAATTGTTGCTG
TGFβ2 (version 1)	305682568c2	Forward	CCCCGGAGGTGATTTCCATC
		Reverse	GGGCGGCATGTCTATTTTGTAAA
TGFβ2 (version 2)	305682568c3	Forward	CCATCCCGCCCACTTTCTAC
		Reverse	AGCTCAATCCGTTGTTCAGGC
TGFβR1 (version 1)	195963411c2	Forward	GCTGTATTGCAGACTTAGGACTG
		Reverse	TTTTTGTTCCCACTCTGTGGTT
TGFβR1 (version 2)	195963411c3	Forward	CACAGAGTGGGAACAAAAAGGT
		Reverse	CCAATGGAACATCGTCGAGCA
TGFβR2 (version 1)	133908633c2	Forward	AAGATGACCGCTCTGACATCA
		Reverse	CTTATAGACCTCAGCAAAGCGAC
TGFβR2 (version 2)	133908633c1	Forward	GTAGCTCTGATGAGTGCAATGAC
		Reverse	CAGATATGGCAACTCCCAGTG
TNC (version 1)	340745336c2	Forward	GCCCCTGATGTTAAGGAGCTG
		Reverse	GGCCTCGAAGGTGACAGTT
TNC (version 2)	340745336c3	Forward	AGGGCAAGTGCGTAAATGGAG
		Reverse	TGGGCAGATTTCACGGCTG
JUN (version 1)	44890066c1	Forward	TCCAAGTGCCGAAAAAGGAAG
		Reverse	CGAGTTCTGAGCTTTCAAGGT
JUN (version 2)	44890066c2	Forward	AACAGGTGGCACAGCTTAAAC
		Reverse	CAACTGCTGCGTTAGCATGAG
MCP1	4506841a1	Forward	CAGCCAGATGCAATCAATGCC
		Reverse	TGGAATCCTGAACCCACTTCT
TGFA	345842399c1	Forward	AGGTCCGAAAACACTGTGAGT
		Reverse	**AGCAAGCGGTTCTTCCCTTC**

Table 6: Continued

EGFR (version 1)	41327735c2	Forward	CCCACTCATGCTCTACAACCC
		Reverse	TCGCACTTCTTACACTTGCGG
EGFR (version 2)	41327735c3	Forward	TTGCCGCAAAGTGTGTAACG
		Reverse	GTCACCCCTAAATGCCACCG
IL2 (version 1)	28178861a2	Forward	TCCTGTCTTGCATTGCACTAAG
		Reverse	CATCCTGGTGAGTTTGGGATTC
IL2 (version 2)	28178861a1	Forward	AACTCCTGTCTTGCATTGCAC
		Reverse	GCTCCAGTTGTAGCTGTGTTT
IL6 (version 1)	224831235c1	Forward	ACTCACCTCTTCAGAACGAATTG
		Reverse	CCATCTTTGGAAGGTTCAGGTTG
IL6 (version 2)	224831235c2	Forward	CCTGAACCTTCCAAAGATGGC
		Reverse	TTCACCAGGCAAGTCTCCTCA
CXCL10	323422857c1	Forward	GTGGCATTCAAGGAGTACCTC
		Reverse	TGATGGCCTTCGATTCTGGATT
MMP1	221004045	Forward	ACACCTCTGACATTCACCAAG
	221004046	Reverse	ATGAGCCGCAACACGATG
ACTA2 (version 1)	16305849	Forward	CAGATGTGGATCAGCAAACA
	221004048	Reverse	TGGCTAGGAATGATTTGGAA
ACTA2 (version 2)	22100451	Forward	GTGTGTGACAATGGCTCTGG
	22100452	Reverse	TGGTGATGATGCCATGTTCT
VIM	221004949	Forward	CAGGAGTCCACTGAGTACCG
	16305849	Reverse	CGGCCAATAGTGTCTTGGTA
PDL1	16184116	Forward	TCACTTGGTAATTCTGGGAGC
	217698287	Reverse	CCTTGAGTTTGTATCTTGGATGCC

Appendix F - Cell Lines

Table 7: Primary cells and cell lines

Sample ID	SOURCE	Mutational status	Site of resection	Pre-op Treatment	Age/sex	Status
TP-12 293	UPCI	$BRAF^{V600E}$	Brain, metastatic	Vemurafenib	53, NA	Deceased
TP-0004	AHN	NA	Skin, cutaneous	PD1	85, M	Alive
TPF-50236 Adjacent	This study	NA	Skin, cutaneous	unknown	73, M	unknown
TPF-50236 Distant	This study	NA	Skin, cutaneous	unknown	73, M	unknown
TPF-50236 Adjacent	This study	NA	Skin, cutaneous	unknown	36, M	unknown
TPF-50236 Distant	This study	NA	Skin, cutaneous	unknown	36, M	unknown

Cell Line	SOURCE	PRODUCT #	Mutational status	Accession	Age/sex
A375	ATCC	CRL-16919	$BRAF^{V600E}$ CDKN2A	CVCL_0132	54, F
WM983A	Wistar Institute, Rockland	WM983A-01-0001	$BRAF^{V600E\ (+/-)}$	CVCL_6808	54, M
WM983B	Wistar Institute, Rockland	WM983B-01-0001	$BRAF^{V600E\ (+/-)}$; TP53	CVCL_6809	54, M
WM1158	Wistar Institute, Rockland	WM1158-01-0001	$BRAF^{V600E\ (+/-)}$	CVCL-6785	NA, M
THP-1	ATCC	TIB-202	$NRAS^{G12R\ (+/-)}$; TP53 *del*	CVCL_0006	1, M

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