Stromal Promotion of Metastasis by Erythroid Differentiation Regulator 1

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Microbiology and Immunology.

Chapel Hill

2010

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Abstract

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Stromal cells and the various factors they release play an important role in the promotion of tumor growth and metastasis. The construction of a supportive tumor stroma involves the recruitment and activation of multiple cell types. CC-chemokine receptor 5 (CCR5) enhances the ability of pulmonary mesenchymal cells (PMCs) to promote metastasis of melanoma cells to the lung, by inducing PMC migration and altering gene expression. In a screen of CCR5-depedent gene expression changes during early metastatic colonization, Erythroid differentiation regulator 1 (Erdr1) was identified as a potential pro-metastatic factor. While its biochemical mechanism of action has not been fully characterized, Erdr1 has been shown to act as a survival factor for several types of hematopoietic cells. The role of Erdr1 in stromal promotion of metastasis was investigated, and Erdr1 expression in both mouse and human cells was characterized. The results indicate that Erdr1 is a highly conserved gene which is expressed in mouse PMCs upon stimulation of CCR5, and which promotes metastasis by enhancing stromal cell survival. This represents a novel mechanism by which chemokine signaling in stromal cells influences the formation of metastasis, and establishes Erdr1 as a potentially important regulator of stromal cell processes in both mice and humans.

Acknowledgements

I would like to thank my mentor, Dr. Jonathan Serody, for guiding my graduate education and advising me in the direction of this project. I thank Dr. Hank van Deventer for also playing an active role in my mentorship, collaborating on experiments, and generally inspiring my scientific curiosity on this topic. I thank my committee, which includes the two above individuals as well as Dr. Jenny Ting, Dr. Glenn Matsushima, and Dr. Yisong Wan, for discussion and guidance. I thank the past and present members of the Serody and Kirby labs for their daily technical assistance and scientific discussion. In particular, I thank Shelly West for her direct experimental contributions and Dr. Joseph Burgents and Dr. Michael Carlson for assistance in brainstorming and data interpretation. Dr. Qing Ping Wu has also directly contributed essential experimental work to this project. I would like to thank others who have contributed technical expertise, including Dr. Deborah Taxman, Dr. Beckley Davis, and Dr. Patricia Ropp. I am grateful to Dr. Eugene Orringer and the UNC MD/PhD program for supporting my medical education and giving me the opportunity to pursue a career as a physician scientist. And I especially thank my wife, Kristen, who since high school has greatly benefitted my personal and professional development through constant advice, support, and encouragement. I would like to express my sincere gratitude to all of these individuals, without whom this work would not have been possible.

Table of Contents

List of Tables and Figures

List of Abbreviations

α-SMA: α smooth muscle actin

A: Adenine

APC: Antigen presenting cell

ATCC: American Type Culture Collection

BAC: Bacterial artificial chromosome

C: Cytosine

CAF: Cancer-associated fibroblast

CBP: CREB binding protein

CCL2: CC-chemokine ligand 2, aka monocyte chemotactic protein 1

CCL21: CC-chemokine ligand 21, aka secondary lymphoid-tissue chemokine 21

CCL3: CC-chemokine ligand 3, aka macrophage inflammatory protein 1α

CCL4: CC-chemokine ligand 4, aka macrophage inflammatory protein 1β

CCL5: CC-chemokine ligand 5, aka regulated on activation normal T cell expressed and secreted

CCR5: CC-chemokine receptor 5

CCR9: CC-chemokine receptor 9

CD34: Cluster of differentiation 34

CD4: Cluster of differentiation 4

CD45: Cluster of differentiation 45, aka protein tyrosine phospatase receptor type C

CD8: Cluster of differentiation 8

CD90: Cluster of differentiation 90, aka Thy1

cDNA: Copy deoxyribonucleic acid

Clca3: Chloride channel calcium activated 3

CLL: Chronic lymphocytic leukemia

CML: chronic myelogenous leukemia

CMV: Cytomegalovirus

CNS: Central nervous system

Co-IP: Co-immunoprecipitation

CREB: Cyclic adenosine monophosphate response element binding

CTGF: Connective tissue growth factor

CTL: Cytotoxic T lymphocyte

CXCL12: CXC-chemokine ligand 12, aka stroma-derived factor 1α

DMEM: Dulbeco's Modified Eagle's Medium

ECM: Extra-cellular matrix

EF-1α: Elongation factor 1α

Erdr1: Erythroid differentiation regulator 1, aka Edr1

EST: Expressed sequence tag

FAP: Fibroblast activation protein

FCS: Fetal calf serum

FGF-2: Fibroblast growth factor 2, aka basic fibroblast growth factor

FISH: Fluorescent *in situ* hybridization

FSP-1: Fibroblast specific protein, aka S100A4

G: Guanine

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

G-CSF: Granulocyte colony stimulating factor

GFP: Green fluorescent protein

HGF: Hepatocyte growth factor

HIV: Human immunodeficiency virus

HSC: Hematopoietic stem cell

IDO: Indolamine 2-3 dioxygenase

IFN-γ: Interferon γ

IGFBP-5: Insulin-like growth factor binding protein 5

IL-12: Interleukin-12

IL-13: Interleukin-13

IL-4: Interleukin-4

IL-6: Interleukin-6

LLC: Lewis lung carcinoma

LPS: Lipopolysaccharide, aka endotoxin

M2: Type II macrophage

M-CSF: Monocyte colony stimulating factor

MDSC: Myeloid-derived suppressor cell

MEF: Murine embryonic fibroblasts

Mid1: Midline 1

MMP: Matrix metalloprotease

MOI: Multiplicity of infection

MSC: Mesenchymal stem cell

NK cell: Natural killer cell

NLC: Nurse-like cell

ORF: Open reading frame

PBMC: Peripheral blood mononuclear cells

PBS: Phosphate-buffered saline

PDGF: Platelet-derived growth factor

PKB: Protein kinase B

PMC: Pulmonary mesenchymal cell

RACE: Rapid amplification of cDNA ends

RAGE: Receptor for advanced glycation end-products

RNA: Ribonucleic acid

RT-PCR: Reverse transcription polymerase chain reaction

SDHα: Succinate dehydrogenase α

SDS-PAGE: Sodium dodecalsulfate polyacrylimide gel electrophoresis

SEM: Standard error of the mean

shRNA: Short hairpin ribonucleic acid

siRNA: Short interfering ribonucleic acid

SOCS-3: Suppressor of cytokine signaling 3

T: Thymine

TAF: Tumor-associated fibroblast

TAM: Tumor-associated macrophage

TGF-β1: Transforming growth factor β

Th1: T helper lymphocyte, type I

Th2: T helper lymphocyte, type II

TLR: Toll-like receptor

Tna: Tetranectin, aka C-type lectin domain 3 B1

TRAIL: Tumor necrosis factor related apoptosis inducing ligand

T_{reg}: Regulatory T lymphocyte

U: Uracil

UTR: Untranslated region

VEGF: Vascular endothelial growth factor

WT: Wild type

Chapter 1
Background and Significance

I. Abstract

The idea that cell types other than tumor cells play a role in cancer pathogenesis has been described by a number of investigators over the past 150 years, but a detailed understanding of the complex interactions between tumors and their stroma has only recently begun to emerge. The supportive microenvironment surrounding a tumor is created when mesenchymal cells, immune cells, endothelial cells and their precursors are recruited and then undergo a phenotypic change to support tumor growth. Stromal cells also promote metastasis, whether from the tumor-adjacent stroma or from the distant target organ. A number of different secreted cytokines and chemokines are involved in this tumor-stroma crosstalk, and investigation of gene expression by stromal cells may unveil novel pharmacological targets. We have previously found that expression of a chemokine receptor, CCR5, by pulmonary mesenchymal cells promoted metastasis to the lung. Our data implicate Erythroid differentiation regulator 1 (Erdr1) in this process. Erdr1 has been found to induce hemoglobin synthesis and to act more generally as a survival factor. Little has been published on this gene, but microarray studies indicate the potential breadth of its function. It is associated with progenitor cells, stromal cells, immune cells, and possibly

development. Our data suggest that the production of Erdr1 by stromal cells is important in their ability to promote metastasis and further that Erdr1 may be critical in the survival of stromal cells. This suggests that a better understanding of the mechanisms of Erdr1 action and methods to inhibit this protein may have clinical relevance in cancer therapy.

II. History of the Concept of Tumor-Stroma Interaction

The analogy of seed and soil.

In 1889, the British surgeon Stephen Paget wrote of metastasis, "If the remote organs in such a case are all alike passive and, so to speak, helpless… then the distribution of cancer throughout the body must be a matter of chance. But if we can trace any sort of rule or sequence in the distribution of cancer, … then the remote organs cannot be altogether passive or indifferent as regards embolism."[1] His survey of 735 breast cancer patient autopsies indeed demonstrated statistical correlations between cancer types and their preferred sites of metastasis. To use Paget's analogy, there might therefore be characteristics of the "soil" which are differentially beneficial or detrimental for successful colonization by metastatic "seeds." Identification and therapeutic manipulation of these characteristics could advance the treatment of malignancy.

In the years since Paget's "Seed and Soil Hypothesis," many soluble factors released by different non-tumor cell types have been found to influence metastasis, providing an increasingly detailed understanding of the "soil." These findings are not limited to sites of secondary growth; the tissue surrounding the primary tumor is clearly involved in tumorigenesis and metastasis as well. A complete picture of cancer pathogenesis must include the complex microenvironment of reciprocal interactions between genetically transformed cells and the multiple other cell populations they encounter.

Stroma is a Greek word meaning "bed," and is used to denote the supportive connective tissue of an organ. Normal tissue stroma consists of mesenchymal cells, specifically quiescent fibroblasts, the extracellular matrix (ECM) they produce, small blood

vessels, and various types of immune cells. In the "tumor stroma" immediately surrounding tumors all of the above components are present, but in a modified state.

Tumor stroma and normal stroma have differential effects on tumor cells

In a 1953 description of 4 clinical cases of skin carcinoma with varying histologies, Pinkus noticed that they differed in their position relative to the dermal stroma. He hypothesized that this reflected different direct effects on the tumor by the stroma.[2] His hypothesis was strengthened by van Scott and Reinertson, who elegantly demonstrated that stroma was required for maintenance of epidermal cell type, by autologous transplantation of epithelial cells to distant areas of the skin or mucosa with or without stroma. They asked, "Since the structure of epithelium seems to be so governed by the supporting stroma, may not the different histomorphologic types of basal cell tumors reflect the influence of a particular stroma, rather than indicate that the tumor originated from any particular epithelial tissue such as hair roots or epidermis?"[3]

Evidence for direct tumor support by the stroma was provided by Cooper and Pinkus in 1977, when they reported that rat basal cell carcinomas transplanted into normal uterine walls of congenic rats gradually acquired normal epidermal histology.[4] Similarly, it was found in 1990 that Dunning prostatic adenocarcinoma cells exhibited glandular differentiation when implanted with mesenchymal cells from developing male urogenital tracts.[5] Furthermore, normal breast fibroblasts, but not tumor-associated breast fibroblasts, were found to inhibit proliferation of a human breast carcinoma line.[6] By demonstrating that normal stromal cells could limit or reverse tumorigenesis, these studies highlighted the dependence of tumors on a supportive stroma.

Supportive stroma associated with breast cancer biopsies was first described by Bano *et al*. as cells adherent in culture that stimulated the attachment and proliferation of carcinoma cells.[7] Using a similar approach, Gerald Cunha and colleagues extended these findings in 1999, showing that cancer-associated fibroblasts (CAFs) isolated from human prostate carcinoma biopsies increased the proliferation of transformed prostate epithelial cells, reduced apoptosis, and promoted tumorgenesis. Normal prostate fibroblasts did not have this effect, nor did CAFs have this effect on normal prostate epithelium.[8]

Some types of stromal cells not isolated from tumors were also found to nevertheless support pathogenesis. Picard *et al*. reported that cultured embryonic fibroblasts promoted the growth of subcutaneous implanted tumors, pointing out that these cells might be quite different than normal organ-resident fibroblasts.[9] Noting that tumor stroma often histologically resemble the granulation tissue found in wounds, Dvorak described tumors as "wounds that do not heal," proposing that the processes underlying wound healing are also supportive for tumors.[10] Directly testing this hypothesis, Dingemans *et al*. found that rat colon adenocarcinoma implanted in pre-induced skin wounds acquired a more de-differentiated and invasive morphology than those implanted in normal skin.[11]

Together these studies established the importance of the stroma on the progression of neoplastic disease. Simultaneous with this work, and with more frequency in the last two decades, the various specific roles of the tumor stroma have been elucidated. These roles include: angiogenesis, provision of survival and growth factors, immune modulation, and promotion of metastasis. Tumors themselves have been found to induce such stromal changes, which can be thought of as integral to the process of tumor development.

III. Construction of Tumor Stroma by Cell Recruitment and Activation

Mesenchymal cells are recruited to tumor stroma.

Myofibroblasts are activated fibroblasts that are defined by their expression α-smooth muscle actin (α-SMA). They also express fibroblast activated protein (FAP), cytokines and chemokines, ECM modification proteins, and contractile fibers. They were first described histologically by Gabbiani *et al.* in wound tissue[12] but they are also apparent in fibrotic lesions as well as tumor stroma. [13, 14] When myofibroblasts are isolated from tumors they are often referred to as cancer-associated fibroblasts (CAFs) or tumor-associated fibroblasts (TAFs), though it should be noted that various groups use these terms whether or not the expression of α-SMA was verified.

The sources of myofibroblasts in wound tissue and tumor stroma have long been hypothesized and debated, but it now seems clear that multiple sources are possible. Fibroblasts can migrate into wound tissue from the surrounding normal tissue[15]. Nearby epithelial cells may be transformed into fibroblasts, in a process known as epithelial to mesenchymal transition (EMT).[16-18] CAFs can also be derived from tissue resident endothelial cells.[19]

There is also recent evidence that tissue myofibroblasts can be derived from bone marrow cells. The hematopoietic origin of cells can be tested by generating bone marrow chimeric mice in which bone marrow from donor mice expressing a certain marker is transplanted into irradiated recipient mice lacking that marker. Over six to eight weeks, bone marrow-derived cells will convert from recipient to donor origin, indicated by their possession of the marker used. This has been demonstrated for myofibroblasts surrounding pancreatic tumors[20, 21], in Lewis lung carcinoma (LLC) stroma[22], in graft vs. host

disease mediated intestinal inflammation[23], in pulmonary fibrosis[24], in liver fibrosis[25], and in cutaneous wound healing[26, 27]. Furthermore, the percentage of cells in the stroma of pancreatic tumors that were bone marrow-derived correlated strongly with the size of the stroma.[28]

Experimental evidence largely indicates that fibrocytes, as well as hematopoietic stem cells and mesenchymal stem cells, are the circulating myofibroblast precursors that migrate into reactive stroma. The initial description of fibrocytes and their role in wound healing was reported by Bucala *et al*.[29]. They described circulating cells expressing CD45, CD34, vimentin, and collagen I, which were recruited to wound sites where they differentiated into myofibroblasts and contributed to fibrotic scar formation.[29] Mori *et al*. later demonstrated that fibrocytes recruited to wound sites gradually acquired expression of α-SMA.[30]

$CD14^+$ monocytes can differentiate into fibrocytes *in vitro* given T cell contact, and then differentiate further into myofibroblasts, both of which processes are enhanced by transforming growth factor β (TGF-β1).[31, 32] The T helper cell type 2 (Th2) polarizing cytokines IL-4 and IL-13, previously found to be associated with lung fibrosis, induce differentiation of monocytes into fibrocytes *in vitro*, while the T helper cell type 1 (Th1) cytokines IL-12 and IFN-γ inhibit it.[33]

Recently Darrel Pilling and colleagues have defined human fibrocytes as $CD45RO^+$, $25F9^+$, $S100A8/9^+$, and PM-2K-, by testing a panel of markers and validating cell type by a combination of *in vitro* differentiation and microscopic examination. The expression of 25F9 distinguishes them from their monocyte precursors, and lack of PM-2K distinguishes them from macrophages. According to Pilling *et al*., after differentiation of fibrocytes into fibroblasts, CD45RO and S100A8/9 expression is lost, and CD90 (Thy1), fibronectin,

hyaluronan, and TE-7 are expressed,[34] though other studies have shown that only a portion of the fibroblast population expresses Thy1.

If fibrocytes are derived from monocytes *in vitro*, this would imply that some fibroblasts *in vivo* ultimately arise from hematopoietic stem cells (HSCs). Indeed, HSCs gave rise to fibroblasts in an *ex vivo* model[35], and to tumor-infiltrating fibrocytes *in vivo*.[36] Mesenchymal stem cells (MSCs), however, have also been proposed as sources for tumor stromal fibroblasts.[37-39] These cells are often thought of as residing in the bone marrow and occasionally circulating in the periphery, but it should be noted that they are theoretically present in any mesenchymal tissue, and so they do not necessarily originate in the bone marrow.

It has been recently questioned whether MSCs represent a distinct cell type from fibroblasts.[40] In fact, MSCs and fibrocytes have many similarities as well, except that MSCs lack the hematopoietic markers CD45 and CD34. It should also be noted that all stromal cells in tissues vary in their expression of α-SMA, vimentin, collagen I, and S100A4, with different populations overlapping in expression of one or more of the markers, indicating that they are heterogeneous and possibly dynamic.[41, 42]

Fibrocytes have been shown to contribute to myofibroblast-mediated pathologies other than cancer, including lung fibrosis[43-48], dermal fibrosis[30, 49], asthma[50-52], and ischemic cardiac myopathy[53]. Migration of fibrocytes into these lesions is mediated by the inflammatory chemokines CCL2[30] and CCL3[45], the homeostatic chemokines CCL21[31] and CXCL12[44], and other factors such as insulin-like growth factor binding protein-5 (IGFBP-5)[48], and adenosine[49].

Activated fibroblasts promote tumor growth

When fibrocytes enter the stroma in any of these processes, they differentiate into fibroblasts and then are activated to become myofibroblasts. Activation of fibroblasts, signified by induction of α-SMA, and their subsequent promotion of tumor growth can be achieved through stimulation by TGF-β1[54-57], IL-4 and IL-13[58], signaling through TLR2, TLR4, and TLR7[59], and possibly through excessive cell contact.[60] The ability of activated fibroblasts to support tumor growth is further enhanced by stimulation of platelet-derived growth factor (PDGF) receptors[61-66]. It has also been found that factors released by tumor cells can induce MSCs to differentiate into myofibroblasts,.[39, 67]

The fibroblast-derived factors mediating tumor growth have been partially defined. In an early co-culture experiment, Valverius *et al*. found that breast fibroblasts supported anchorage independent growth of transformed breast epithelial cells, through secretion of transforming growth factor α (TGFα) and fibroblast growth factor 2 (FGF-2).[68] Other groups have found that co-injection of tumor cells with fibroblasts enhanced tumor growth.[69-71] Other factors include matrix metalloproteases (MMPs)[72-74], which degrade the ECM, hepatocyte growth factor (HGF)[75-79], TGFα[79], FGF-2[56, 80], CXCL12[81-84], connective tissue growth factor (CTGF)[57], IL-6[38, 85, 86], S100A4[87], and osteopontin[64]. Through release of these factors, recruited and activated fibroblasts directly promote tumor growth.

Immune escape involves recruitment and suppression of immune cells.

Rudolf Virchow hypothesized in 1863 that tumors themselves were caused by chronic inflammation after observing the frequent co-localization of tumors and inflammatory cells.[88] As mentioned previously, the resemblance of tumor stroma to granulation tissue prompted Dvorak to refer to tumors as "wounds that do not heal." [10] Both Dvorak and Virchow partly based these views on histological evidence of immune cell infiltration of tumor stroma. Certainly some immune cells infiltrating tumors have anti-tumor functions, particularly $CD8^+$ cytotoxic T lymphocytes (CTL) and natural killer (NK) cells. It is widely hypothesized that many subclinical neoplasms are cleared by the immune system before they can cause significant disease (for a recent introductory review on anti-tumor immunity, see [89]). However, the accumulation of suppressed immune cells often counteracts tumor rejection, leading to the need for therapeutic intervention.

In 1983, Montovani and colleagues demonstrated that tumor conditioned media induced chemotaxis of macrophages, and the number of tumor-associated macrophages (TAMs) in the tumor stroma *in vivo* correlated with the ability of a given tumor cell line to condition media for macrophage chemotaxis *in vitro*.[90] In 1985 Taylor and Black reported that B16-F10 melanoma-derived membrane microvessicles inhibited the activation of macrophages.[91] TAMs have a suppressive "Type 2" macrophage (M2) phenotype, characterized by secretion of IL-10, IL-1R antagonist, and chemokines for Th2 cells and regulatory T cells (T_{reg}).[92] TAMs have been found to secrete TGF-β1, resulting in suppression of activated T cell proliferation[93] and to promote the expression of pro-myofibroblast and pro-angiogenesis cytokines by tumor cells.[94]

Another immune cell found in tumor stroma is the myeloid-derived suppressor cell (MDSC). These are immature myeloid cells of either monocytic or granulocytic lineages, and are dramatically expanded in the spleens and peripheral blood of tumor-bearing animals[95, 96] and patients[97, 98]. T_{reg} are also found in the tumor stroma and contribute to immune suppression. Among other functions, they have been found to contribute to the suppressive phenotype of TAMs.[99] T_{reg} also partially mediate the suppressive effect of MDSCs.[100] Reciprocally, MDSCs inhibit $CD4^+$ and $CD8^+$ effector T cell function.[101]

Fibroblasts in the tumor stroma recruit and influence the suppressed phenotype of these cell types. S100A9, often expressed by tumor stroma, contributes to the accumulation of MDSCs in tumor-bearing mice, as well as the reduction of dendritic cells.[102] Stroma-derived TGFβ1, has many immune suppressive effects, including the polarization of adaptive T_{reg}[103], recruitment of TAMs [104], recruitment of MDSCs[105], and possibly polarization of suppressive neutrophils[106]. Stroma-derived CCL2 recruits monocytes[107] and T cells[108], and enhances to pro-tumor activity of TAMs[109, 110]. Tumors have been found to induce fibroblasts to express the chemokines CXCL1, 2, and 5, and CCL3, as well as the cytokines IL-11 and IL-1β.[111, 112]

Fibroblasts also inhibit T cell activation[113, 114] and antigen presenting cell (APC) activity, particularly through the expression of indolamine 2-3 dioxygenase (IDO).[115, 116] Activated fibroblasts have been shown to induce FasL-mediated death of T cells.[117] MSCs, which are phenotypically similar to fibroblasts, also inhibit T cell activation[118], possibly through IGF binding proteins[119] or IDO[120]. Moreover, proliferation of previously activated T and B cells can be inhibited by MSCs[121], as can dendritic cell activation[122-124]. Immunosuppression mediated by mesesnchymal cells is relevant to

tumor immunity, since injection of MSCs allowed growth of B16-F10 melanoma cells in allogeneic mice.[125, 126]

Fibroblasts and recruited monocytes contribute to angiogenesis.

The formation of new blood vessels to support tumor growth is another important function of the stroma. In 1971, Folkman and colleagues, hypothesized that angiogenesis was crucial for tumor growth, and proposed targeting this process as a tumor treatment strategy.[127] As with other stromal processes, angiogenesis involves the recruitment of multiple cell types. After transplant with green fluorescent protein (GFP) expressing HSCs, GFP positive endothelial cells were found in the stroma of Lewis Lung tumors.[22] Other studies have shown that both monocytes[128] and mesenchymal cells[128, 129] are recruited to the vascular regions of tumor stroma. Circulating endothelial cells are also recruited[83, 130] and this may in part be mediated by fibroblast-derived CXCL12[83]. The recruitment of bone marrow-derived fibroblasts and bone marrow-derived endothelial cells appears to be important, since a survey of 10 different tumor types found that it is correlated with tumor growth rate.[131]

Vascular endothelial growth factor (VEGF) was first identified by Dvorak and colleagues in ascites fluid from tumor-bearing guinea pigs, and in multiple tumors and tumor lines.[132] Six years later, two separate groups found that it promoted endothelial cell proliferation[133, 134], and also induced monocyte activation and migration[135]. Today VEGF is recognized as a major contributor to tumor angiogenesis, and blockade of VEGF in cancer patients has been shown to prolong survival when combined with chemotherapy (for a recent review, see [136]). Of particular interest here is the work by Lyden *et al*., showing

that tumor angiogenesis depends on VEGFR-mediated recruitment of bone marrow-derived hematopoietic and endothelial cell precursors.[137] TAFs have been shown to express VEGF, particularly the $Thy1^+$ population.[86]

Other fibroblast-produced cytokines previously discussed promote angiogenesis as well, including TGF-β1[138], IL-1α and IL-1β[139], and CXCL12 [140]. In EL4 tumors, which do not respond to anti-VEGF antibody therapy, TAFs were found to express high levels of PDGF-C, a gene closely related to VEGF. When PDGF-C was blocked, angiogenesis and tumor growth was reduced.[141]

Taken together, these studies show that the construction of the tumor microenvironment depends on the recruitment of multiple cell types, which are then stimulated by tumor cells to secrete tumor-supporting factors. While immune cells and vascular endothelial precursors are vital for tumor protection and blood supply, the expression of chemokines and growth factors relevant to all of these processes establishes mesenchymal cells as a central player in tumor growth.

IV. Mesenchymal Cells and Metastasis

Activated fibroblasts at the primary tumor site promote metastasis

In 1987, Stoker *et al*. reported that a fibroblast-derived protein induced scattering of epithelial cells *in vitro*, and named the protein scatter factor.[142] They later found that it induced the invasion of human carcinoma lines *in vitro*, and that it was identical to the previously discovered protein HGF.[143, 144] They hypothesized that *in vivo*, HGF might enhance the metastatic potential of tumor cells.

Indeed, HGF was found to be expressed in many human malignant tumors [75], and *in vivo* animal models showed that their hypothesis was correct.[145, 146]. The evidence for expression of HGF by tumor stromal cells has been discussed above, but it is of note that three different tumor lines induced HGF production by fibroblasts in co-culture, and this in turn promoted the invasiveness of the tumor cells.[76]

Other molecules secreted by stroma have been found to promote the progression of tumors to a metastatic phenotype, including MMP-9[147], monocyte colony stimulating factor (M-CSF)[148], and TGF-β1[105]. In a reciprocal enhancement of motility, tumor-derived microvessicles induced fibroblast migration, and fibroblasts exposed to tumor microvessicles induced migration of PC3 cancer cells in culture.[149]

Chemokines are known to induce chemotaxis, but they can also directly enhance the metastatic potential of tumors. MSCs increased the metastatic potential of four separate breast cancer lines *in vivo*, and this was found to depend on MSC expression of CCL5 (RANTES) and tumor cell expression of CCR5.[150] Furthermore, CXCL12 and CCL21 increase the metastatic potential of tumor cells by preventing anoikis, the process of cell death stimulated by lack of ECM contact.[151]

Chemokines direct metastatic cells to secondary sites

Human breast cancer cells were found to express CXCR4 and CCR7, the expression of whose ligands, CXCL12 and CCL21, were found to be higher in the tissues to which breast cancer metastasizes. Human melanoma cells, which have similar metastatic target organs, also expressed these two receptors, in addition to CCR10, whose ligand CCL27 is expressed in skin. Antibody neutralization of CXCR4 in SCID mice injected with human breast cancer cells reduced metastasis to the lungs and lymph nodes.[152] B16-F10 melanoma cells were found to migrate towards fibroblast-derived proteins S100A8 and S100A9 via the RAGE receptor.[153]

CXCR4 was also expressed by multiple patient samples and cell lines of pancreatic tumors, and expression correlated with metastasis.[154] Forced over-expression of CXCR4 in B16-F10 cells increased metastasis to the lung and skin.[155] CXCR4 expression is upregulated by hypoxia in tumor cells[154] and many normal cell types[156], suggesting a process in which cells in the middle of a tumor that has outgrown its blood supply might prepare for metastasis. Forced expression of CCR7 was shown by Wiley *et al*. in 2001 to drive melanoma metastasis to lymph nodes, which express CCL21.[157] It was recently shown that T cell acute lymphocytic leukemia brain metastasis also depends on migration via CCR7.[158]

Mesenchymal cell gene expression determines the "fertility" of the "soil"

Regardless of whether the construction of supportive stroma in distant organs must occur prior to colonization, there is evidence that the stromal gene expression does influence metastasis. Many of the stroma-derived factors that support primary tumor growth also support the development of metastases in experimental models, including HGF[159], IL-6[85], and MMP-9[160]. Some of these factors, or genes regulating them, appear to be particularly important for stromal promotion of metastasis.

For example, S100A4, expressed by activated fibroblasts[161], has long been appreciated as a tumor cell-expressed gene promoting metastasis through induction of MMPs.[162-165] However, it has more recently been demonstrated that S100A4 expressed by other cells can also promote metastasis.[166, 167] In fact, S100A4 deficient mice were found to be completely resistant to breast cancer metastasis to the lung, and this was reversed by transfer of WT fibroblasts.[87] Saha *et al*. recently provided evidence that S100A4 and S100A8 were negatively regulated by utereoglobin (UG). UG knockout mice expressed more of these genes in lung tissue than WT mice, and developed more metastases.[153]

Conversely, a tumor cell-derived proteoglycan, versican, stimulated monocytes through TLR2 to produce IL-6 and TNF, and this promoted metastasis.[168] Metastasis of colon cancer was found to be dependent on stromal expression of PDGFR.[65, 85, 87, 153] CXCL12 and TNF-related apoptosis inducing ligand (TRAIL) expression in the bone marrow has been found to support survival of breast cancer metastases to the bone marrow.[169] These examples illustrate that target organ gene expression directly effects the process of metastasis.

In 2005 our group reported that CCR5$^{-/-}$ mice develop fewer metastases than wild type (WT) mice.[170] This was determined not to be dependent on expression of CCR5 on hematopoietic cells, as reconstitution of irradiated CCR5$^{-/-}$ mice with WT bone marrow did not result in increased metastasis formation. Rather, transfer of WT pulmonary mesenchymal cells (PMCs) to CCR5$^{-/-}$ mice prior to tumor cell injection resulted in numbers of metastases similar to WT mice, indicating that expression of CCR5 increased the ability of PMCs to support metastasis.[170]

Further work demonstrated that fibrocytes expressed CCR5, and migrated into the lungs more efficiently than CCR5$^{-/-}$ fibrocytes after transfer. Once in the lung, WT fibrocytes increased expression of MMP-9, which was found *in vitro* to be stimulated by the CCR5 ligand CCL5. Multiple other chemokines were also demonstrated to be expressed in resting lungs, indicating that fibrocytes could migrate into lungs in a chemokine specific manner under normal conditions, i.e. before metastatic colonization. In fact, 15-20% of resting lung PMCs cultured *ex vivo* were found to be CD45^{+} fibrocytes, which partly explained how these bone marrow-derived cells could promote metastasis in the previous study even after irradiation.[171]

The results of these studies were confirmed and extended by Wu *et al*., who also found reduced metastases in CCR5$^{-/-}$ mice, as well as mice deficient in the CCR5 ligand CCL3. In addition, they found increased migration of monocytes and fibroblasts into the lungs of WT mice after tumor injection, as well as increased monocyte-derived MMP-9 and fibroblast-derived HGF expression, relative to CCR5$^{-/-}$ or CCL3$^{-/-}$ mice.[159]

Previous work had demonstrated a delay in tumor growth in CCR5 $^{-/-}$ mice, and these mice responded better to therapeutic vaccine with WT dendritic cells than did WT mice, but

the effect on primary tumor growth without vaccine was not statistically significant.[172] However, the significant effect of CCR5 on metastasis development suggests that the construction of supportive stroma in the secondary organ may depend more on CCR5 signaling than that of primary tumor stroma. The role of CCR5 in metastasis and tumor vaccine response is particularly clinically relevant, since an effective CCR5 inhibitor, Maraviroc, is available and approved for use in preventing human immunodeficiency virus (HIV) infection of T cells.[173]

CCR5 $^{-/-}$ mice were defective in the ability of their PMCs to contribute to pro-metastatic stroma, but the mechanism of this defect was not initially clear. Therefore, a comparative gene expression study was undertaken in WT vs. CCR5$^{-/-}$ lungs soon after injection of malignant melanoma cells, as will be discussed in Chapter 2. One potential pro-metastatic candidate identified was a little-studied gene with no previous connection to metastasis: erythroid differentiation regulator 1 (Erdr1).

V. Background on Erythroid Differentiation Regulator 1

Initial Characterization of Erdr1

In 1992 Dormer and colleagues presented data at the annual meeting of the Society for Hematology and Stem Cells showing that media conditioned by the myelomonocytic leukemia cell line WEHI-3B induced differentiation of an erythoblastic leukemia cell line.[174] As described in a pair of articles in 2004, the responsible factor was isolated and called Erythroid Differentiation Regulator 1 (Erdr1, previously Edr1).[175, 176]

The differentiation of cells in the erythroid lineage results in increased expression of hemoglobin, which can be measured using a benzidine-based colorimetric assay. Dormer *et al*. used this assay to screen a WEHI-3B cDNA library for clones expressing the hemoglobin-inducing factor. The insert sequence of the single positive clone had an open reading frame of 534 bp (177 amino acids), had no homology to other known genes, and was named *Erdr1*. Both recombinant Erdr1 and WEHI-conditioned media were found to induce hemoglobin synthesis over a biphasic dose-response curve. Erdr1 protein was detected in primary murine lymphoma, a murine lymphoblastoid cell line, human G-CSF mobilized $CD34^+$ stem cells, PBMCs from chronic myelogenous leukemia (CML) and B cell chronic lymphocytic leukemia (CLL) patients, and human umbilical cord blood T cells. Immunofluorescense staining of murine erythroleukemia cells and human cord blood T cells revealed that Erdr1 was localized to the cell membrane and appeared to be released in membrane vesicles, though its hemoglobin inducing activity was demonstrated in both the vesicle and soluble fractions of conditioned media.[175]

In a second round of screening of the WEHI-3B cDNA library using the clone from the first screen, a transcript with a slightly longer ORF (630 bp, 209 aa) was identified. Multiple mRNA species were identified by Northern blot, none of which contained either fully spliced ORF. Remarkably, the mRNA sequence with the longer ORF was reported to be identical between human and mouse, when amplified by RT-PCR.[175] The details of the molecular characterization of Erdr1 will be further discussed in Chapter 3.

Dormer and colleagues subsequently tested the effects of Erdr1 on human hematopoietic progenitors and a Burkitt's lymphoma cell line (BL-70). When BL-70 cells are removed from their stromal cell feeder layer, they undergo apoptosis unless they are cultured at very high cell densities. Erdr1 protected BL-70 from apoptosis at low cell density and reduced the cell density necessary for survival. However, this was only true at certain concentrations, and Erdr1 enhanced apoptosis at higher concentrations. Regarding hematopoietic progenitors, such biphasic responses were also seen in erythroblast differentiation and proliferation of primary human erythroid and granulocytic/monocytic progenitors.[176]

A recurring theme in the initial studies of Erdr1 is the association with stress responses. WEHI-3B cells expressed and released more Erdr1 after incubation in serum-free media.[175] NIH 3T3 (murine embryonic fibroblasts) released Erdr1 after switching from 10% to 2% serum-supplemented media[175], and after trypsinization[176]. MEL cells released Erdr1 upon transfer to PBS, incubation at room temperature, incubation at 41.5 C, and exposure to H_2O_2.[176] .

Together, these findings suggest that Erdr1 is a widely expressed, highly conserved cell stress-related cytokine that can act as both a survival factor and as an inducer of

differentiation. Dormer *et al*. have hypothesized from its expression in multiple organs and cell types that its function is likely more general than related to the expression of hemoglobin during hematopoiesis. Indeed, the bioinformatics data now available through BioGPS[177] confirms expression in a wide range of tissues and cells, the highest being the female reproductive organs, the spleen, the CNS, and embryonic stem cells (see Appendix I). Further insight into the broad range of the potential roles Erdr1 might play can be attained through a survey of functional genomics databases (see Appendix II). While these data are by their nature preliminary, in the absence of conserved domains or sequence homology to other genes, they provide a valuable framework for the interpretation of studies directly targeting Erdr1.

Erdr1 in Stem Cells and Progenitors

Since Erdr1 was first isolated from myelomonocytic leukemia cells (WEHI-3B) and its effects studied in hematopoietic progenitors, it is not surprising that multiple groups have found its expression to be associated with cells that are less differentiated (App. II, 1-9). Sung *et al*. found it to be 4 to10 -fold higher in hematopoietic stem cells and progenitors than in granulocytes[178] (App. II, 2-4) and Liadaki *et al*. found it more than 3 -fold higher in bone marrow side population cells identified by Hoechst 33342 stain[179] (App. II, 6). Silva-Vargas *et al*. found that induction of β-catenin conditional transgenic mice on the skin, which induces de-differentiation of hair follicles, also induced a roughly 13 -fold increase in Erdr1.[180] (App. II, 1) Conversely, differentiation of C2C12 myoblasts to myotubes was associated with a roughly 2 -fold decrease in Erdr1 expression.[181, 182] (App. II, 8-9)

The association of Erdr1 expression with less differentiated hematopoietic cells in expression arrays (App. II, #2-4, 6) led Denault *et al*. to include it in a functional *in vivo* screen of potential promoters of HSC self-renewal. 104 candidate genes were transduced into HSCs, which were then tested in an *in vivo* competitive re-population assay. Erdr1 increased HSC activity by about 4-fold, decreased HSC apoptosis, and increased HSC expansion *in vitro*. *Erdr1*-transduced HSCs isolated from mice in the primary re-population assay were also found to promote secondary re-population in a new group of mice, indicating that its mechanism of action was increasing HSC self-renewal rather than increasing the efficiency of differentiation to pluripotent cells.[183]

Their purpose for doing the screen was to identify a small set of genes which, when transfected together into a terminally differentiated hematopoietic cell, would return it to a HSC phenotype. While several arrays associate Erdr1 with less differentiated cells, there has been no evidence to date that Erdr1 can reverse the differentiation of cells, and a simple HSC pro-survival effect cannot be ruled out as an explanation for their results.

Erdr1 in Stromal Cells

Dormer *et al*. found that the human bone marrow stromal cell line L88/5 released a factor when irradiated that induced MEL hemoglobin synthesis, though they did not definitively demonstrate that this factor was Erdr1.[175] They further showed that recombinant Erdr1 partially substituted for a stromal cell feeder layer in the culture of Burkitt's lymphoma cells.[176] Microarray cDNA analyses too have associated Erdr1 with stroma. Stimulation of MEFs with FGF-2 induced expression of Erdr1. (unpublished study, GEO dataset GDS2421) (App II, 23) Furthermore, the stromavascular fraction of adipose

tissue was found to express 4 to 5 -fold more Erdr1 than the adipocyte fraction.[184] (App II, 17-18)

In conclusion, the hypotheses of Paget in 1889 and Pinkus in 1953 that stroma plays an active role in cancer progression have been validated both generally and specifically regarding certain stromal cell types and factors. The construction of tumor stroma includes the recruitment and activation of mesenchymal cells, both tissue-resident and bone marrow-derived. Tumor stroma construction also involves recruitment of endothelial cell precursors for angiogenesis and of immune cells, both of which also interact with tumors and mesenchymal cells to promote tumor growth and immune escape.

Cells in the stroma promote the progression of tumors to a metastatic phenotype, and once the tumor cells metastasize, cells in distant organ stroma provide a fertile "soil" by attracting them via the production of chemokines, modifying the ECM with MMPs, and supporting their survival and growth in ways similar to primary stroma. The recruitment of stromal cells to the lungs via CCR5 appears to be important for metastasis. Data presented in Chapter 2 will link Erdr1 to this process as well. This gene was isolated for its ability to induce hemoglobin synthesis in erythroblasts, but its expression by stromal cells under stressful conditions and its proven role as a survival factor make it an ideal candidate for contributing to the fertility of Paget's "soil."

Chapter 2
CC-Chemokine Receptor 5 on Pulmonary Stromal Cells Promotes Metastasis through Induction of Erythroid Differentiation Regulator 1

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I. Summary

We have previously shown that pulmonary mesenchymal cells (PMCs) from CC-chemokine receptor 5 (CCR5) deficient mice have a reduced ability to promote the metastasis of B16-F10 melanoma cells. To determine the mechanism for this finding we used cDNA microarrays to compare the gene expression in wild type (WT) and CCR5 $^{-/-}$ lungs after intravenous injection of B16-F10 cells. We found increased expression of Erdr1 in the lungs of WT compared to CCR5 $^{-/-}$ mice in the first 48 hours after injection of tumor cells. This increase was confirmed by RT-PCR. Furthermore, WT PMCs expressed more Erdr1 than CCR5$^{-/-}$ PMCs *in vitro,* and stimulation of PMCs with the CCR5 ligand, CCL4, increased expression of Erdr1. Knockdown of Erdr1 in murine embryonic fibroblasts led to a reduced ability of those cells to promote B16-F10 metastasis in CCR5$^{-/-}$ but not WT mice. *In vitro,* Erdr1 knockdown PMCs had a growth disadvantage compared to PMCs expressing control shRNA, due to increased apoptosis, which was partially reversed by caspase inhibition. Taken together, our data suggest that CCR5 promotes metastasis by supporting PMC survival through induction of Erdr1.

II. Significance

Multiple lines of evidence indicate that stromal cells support the formation of metastasis through the release of extracellular factors. Identification of these factors could reveal novel targets for therapeutic prevention of malignancy. Here, we show that the stimulation of pulmonary mesenchymal cells (PMCs) through CCR5 leads to the expression of a recently characterized gene, Erythroid Differentiation Regulator 1 (*Erdr1*). Furthermore, Erdr1 acts as a stromal cell survival factor which may be critical for the stromal cell promotion of metastases in the lung. This report is the first to link Erdr1 with metastasis, and presents a novel mechanism by which chemokine signaling in stromal cells promotes metastasis.

III. Introduction

Over the past ten years, evidence has emerged highlighting the importance of stromal cells in cancer progression. Cells in the tumor stroma promote tumor growth[185], mediate angiogenesis[185], contribute to an immune-suppressive microenvironment[89], and increase the invasiveness of solid tumors[186]. When tumor cells metastasize, a new supportive stroma is constructed around the metastatic nodule. This requires the secretion of pro-metastatic factors including MMP-9[160], S100A4[87, 153, 167], S100A8[153], and IL-6[85]. The activation and proliferation of stromal fibroblasts and the recruitment of fibrocytes and other bone marrow-derived cells contribute to the formation of the new stroma. For example, platelet-derived growth factor C (PDGF-C) has recently been shown to induce fibroblast activation and recruitment [64], and blockade of its receptor, PDGFR, on fibroblasts reduced metastasis in a model of colon cancer[65].

We have previously shown that mice deficient for CC chemokine receptor 5 (CCR5) developed fewer metastases than wild type (WT) mice, and this was reversed by intravenous transfer of WT pulmonary mesenchymal cells (PMCs) to $CCR5^{-/-}$ mice.[170] We subsequently showed that CCR5 enhanced the ability of injected PMCs to migrate into the lung, but that their persistence there was limited.[171] Furthermore, stimulation of CCR5 induced production of MMP-9 by $CD45^{+}$ fibrocytes, which comprised 15-20% of PMCs, and treatment at the time of tumor injection with an MMP-9 inhibitor reduced metastasis.[171] Others have found that the reduction in metastasis also extended to mice deficient in the CCR5 ligand CCL3, and that WT lungs accumulated more monocytes, T cells, and

fibroblasts than $CCR5^{-/-}$ or $CCL3^{-/-}$ lungs, as well as more production of MMP-9 and HGF.[159] Therefore CCR5 signaling is important for the formation of pro-metastatic stroma. The further investigate the mechanisms for this finding, we compared gene expression in WT vs. $CCR5^{-/-}$ lungs after B16-F10 melanoma cell injection.

Erythroid Differentiation Regulator 1 (Erdr1) has not previously been associated with tumor growth, metastasis, or chemokine signaling. It was first isolated as a cytokine released by the myelomonocytic cell line WEHI-3B that induced hemoglobin synthesis. [175] Further investigation found Erdr1 expression in the murine embryonic fibroblast cell line NIH-3T3, primary murine lymphoma lysates, and human $CD34^{+}$ enriched peripheral blood mononuclear cells (PBMCs).[175] It was found to be released under a number of different conditions of cell stress, and it acted as a survival factor for a Burkitt's lymphoma cell line as well as for erythroblast and granulocyte/monocyte progenitors.[176] When expressed in hematopoietic stem cells (HSCs), Erdr1 similarly promoted their expansion and inhibited apoptosis, as well as promoting their ability to repopulate bone marrow *in vivo*.[183] Erdr1 expression has been correlated with a number of cell types and biological processes by cDNA microarrays, including HSCs[178, 179], stromal cells[184](GEO Dataset GDS2421), brain inflammation and neurodegenration[108, 187-189], innate and adaptive immunity[190-192], and development [193-195].

Here we show a novel function of Erdr1, which is induced after the binding of ligand to CCR5 on stromal cells. This activity results in the promotion of lung metastasis by stromal cells *in vivo* and their enhanced survival *in vitro*. Thus, Erdr1 may be a novel target for the prevention of lung metastasis clinically.

IV. Results

Genes differentially expressed in WT vs. CCR5 $^{-/-}$ lungs

We have previously shown that CCR5$^{-/-}$ mice develop fewer metastases than WT mice. To identify genes which might mediate CCR5-dependant promotion of metastasis, we injected B16-F10 melanoma cells into WT or CCR5 $^{-/-}$ mice intravenously and harvested lung RNA 6, 24, and 48 hours later for Affymetrix expression analysis. cDNA was pooled at each time point from 4 or 5 mice of each genotype. We found 11 candidate genes differentially expressed by WT vs. CCR5$^{-/-}$ lungs at all three time points (Fig. 2.1A). Of these, *Mid1*, *Erdr1*, and *Ccr9* had more than a two-fold higher expression and *Clca3* and *Tna* had a more than 2-fold lower expression in WT compared to CCR5$^{-/-}$ mice. To confirm the expression data found by cDNA microarray, semi-quantitative RT-PCR was performed for the 11 candidate genes using cDNA from each mouse. The expression of most of these genes displayed significant variability (Fig. 2.S1), and of the 5 genes with more than two-fold differential expression by microarray, only the expression of Erdr1 was significantly different by densitometry (Fig. 2.1B) at all time points analyzed. Erdr1 expression was similar in all WT mice and near or below the level of detection for all CCR5$^{-/-}$ mice (Fig. 2.1C). Therefore, we chose to further characterize the role of Erdr1 in the promotion of lung metastasis.

Erdr1 expression in pulmonary mesenchymal cells

Since previous work demonstrated that the reduction of metastases in CCR5 $^{-/-}$ mice can be reversed by the transfer of WT but not CCR5 $^{-/-}$ PMCs [170], Erdr1 expression was

measured in cultured PMCs. By real time RT-PCR, Erdr1 expression in WT PMCs was 6.4 $^{\pm}$ 2.7 -fold higher than in CCR5$^{-/-}$ PMCs (Fig. 2.2A) ($p < 0.001$). The higher production of Erdr1 in WT PMCs was confirmed by Western Blot (Fig. 2.2B). Cultured PMCs contain three different populations of stromal cells, which can be defined by expression of CD45 and Thy1. To determine the expression of Erdr1 by each individual population, WT and CCR5$^{-/-}$ PMCs were sorted for these different populations, and Erdr1 expression was analyzed by real time RT-PCR. WT PMCs from the CD45^{+} (fibrocyte), Thy1^{+}CD45^{-}, and Thy1^{-}CD45^{-} populations all expressed more Erdr1 than their CCR5$^{-/-}$ equivalents (Fig. 2.2C) ($p < 0.005$, $p < 0.05$, $p < 0.005$, respectively). There was also significantly more Erdr1 expression relative to *Sdh*α in WT Thy1^{-}CD45^{-} PMCs than in WT fibrocytes (0.49 vs 0.24, $p < 0.05$) or in WT Thy1^{+}CD45^{-} PMCs (0.49 vs. 0.32, $p < 0.05$). These differences were not seen between the PMC populations from CCR5$^{-/-}$ mice.

Erdr1 was originally cloned by Dormer *et al*. from the myelomonocytic cell line WEHI-3B [175](accession A539223), and the current reference sequence was cloned by the Mammalian Gene Collection Program Team from a murine mammary tumor [196](accession NM_133362). There are a few differences between the sequences from these two different cell types, in both the nucleotide and amino acid sequences. To determine whether PMCs expressed similar transcripts to either of these, we compared the sequence of the cDNA isolated from PMCs of WT animals to that previously found in WEHI-2B or a mouse mammary tumor cell line. As shown in Fig. 2.S2, the ORF in the *Erdr1* consensus sequence isolated from PMCs was 100% homologous to the sequence found in the WEHI-3B cell line (sequence AJ539223).

Induction of Erdr1 expression by CCL4

To determine whether CCR5 signaling induced expression of Erdr1, WT and CCR5 $^{-/-}$ PMCs were stimulated with the CCR5-specific chemokine, CCL4. As measured by real time PCR, Erdr1 was induced by CCL4 at 24 hrs (1.33 -fold $^{\pm}$ 0.06, $p < 0.05$) and 48 hrs (3.36 -fold $^{\pm}$ 0.14, $p < 0.0005$)(Fig. 2.3A) in WT PMCs. Erdr1 was not induced by CCL4 in CCR5 $^{-/-}$ PMCs. The difference between fold Erdr1 induction in WT and CCR5 $^{-/-}$ CCL4 treated PMCs from 3 separate experiments was statistically significant (Fig. 2.3B, $p < 0.05$), indicating that ligand binding to CCR5 up-regulates the expression of Erdr1. We further confirmed these findings by Western blot (Fig. 2.3C). We then tested the capacity of CCL4 to induce Erdr1in sub-populations of PMCs. In these experiments, PMC cultures were sorted by Thy1 and CD45 expression (Fig. 2.3D) after 48 hours of treatment with CCL4. A 14.2 -fold $^{\pm}$ 2.9 increase of Erdr1 expression was seen in WT $CD45^{+}$ fibrocytes, but not in $CCR5^{-/-}$ fibrocytes ($p < 0.01$ for CCL4 vs. media and $p < 0.01$ for WT vs. $CCR5^{-/-}$) (Fig. 2.3E). Other fold differences between populations upon CCL4 stimulation were not statistically significant.

Reduction in Metastasis after Erdr1 Knockdown

To investigate the role of Erdr1 in metastasis we first used murine embryonic fibroblasts (MEFs). We determined that like PMCs, MEFs promoted metastasis in $CCR5^{-/-}$ mice (Fig. 2.4A) and expressed Erdr1 (Fig. 2.S3A&B). We constructed two *HSPG* retroviral vectors carrying different Erdr1-specific short hairpin RNA (shRNA) sequences which we had previously determined effective by transfection (data not shown), as well as one vector

carrying a "No Target" shRNA control (Fig. 2.S3C). By measuring the fluorescence of GFP co-expressed by the vectors, we found that they efficiently transduced MEFs (Fig. 2.4B). These were initially sorted by GFP expression to separate the non-transduced cells. The two Erdr1-targeted shRNA vectors reduced Erdr1 expression by 89.2% $^{\pm}$ 2.5% and 99.5% $^{\pm}$ 2.5% relative to the no target shRNA vector (Fig. 2.S3D). However, the sort-purified Erdr1 knockdown MEFs did not expand *in vitro*, and thus could not be used for *in vivo* studies. As a result, shRNA transduced MEFs were used 48 to 72 hours after transduction without separating GFP^+ from GFP^- cells. Figures 2.4B and 2.4C show a representative culture of Erdr1 knockdown lines with a transduction efficiency of approximately 70% and a knockdown efficiency of 55.9% $^{\pm}$ 1.1% for shRNA #1 and 68.8% $^{\pm}$ 6.5% for shRNA #2. Knockdown of Erdr1 was further confirmed by Western blot (Fig. 2.4D).

In order to test Erdr1 effect on metastasis, $CCR5^{-/-}$ mice were intravenously injected with Erdr1 knockdown MEFs 48 hours prior to injection of B16-F10 melanoma cells. As shown in figure 2.5A, the knockdown of Erdr1 led to a 32.6% and 32.2% decrease in lung metastasis from B16-F10 tumor cells fourteen days after the injection of tumor cells ($p < 0.05$ for both shRNA constructs). The range of transduction efficiency of the MEFs varied for each of these experiments and this could have diminished the effect found after knocking down the expression of Erdr1. Thus, we grouped recipient mice into those in which the transduction efficiency was > 50% and those with a transduction efficiency of < 50%. As shown in Fig. 2.5B, there was a 46.6% and 50.4% reduction in metastasis in the > 50% transduced group ($p < 0.05$ for both) and only a 23.8% and 24.3% reduction in the group in which less than 50% of the cells were transduced, which was not statistically significant. Conversely, when Erdr1 knockdown lines were transferred to WT mice prior to tumor

injection, there was no reduction in metastases compared to the group receiving control shRNA MEFs (Fig. 2.5C), which is similar to our findings transferring PMCs to wild type mice. Thus, these data demonstrate that Erdr1 expression is important for the growth of B16-F10 tumor cells in a metastatic lung model. Our finding that the level of transduction and as a result the knockdown of Erdr1 corresponded to the in vivo reduction of tumor metastases strongly suggests that this finding is not a result of off target effects.

Overexpression of Erdr1 sequence induces cell death.

To confirm our findings using shRNA targeting Erdr1, we sought to overexpress Erdr1 in $CCR5^{-/-}$ PMCs. Therefore, we cloned Erdr1 into a lentiviral vector and tested its function. Two different constructs were generated using a strong promoter from CMV and a weaker promoter E2F1α.

A293T cells, which express very low Erdr1, were transfected first with the expression plasmids alone, without packaging plasmids. As shown in Fig. 2.6A, this increased production of *Erdr1* mRNA by at least 100-fold. A 100-fold increase was also seen in PMCs transduced with either Erdr1 expressing lentivirus (Fig. 2.6B). However, this magnitude of expression rapidly led to cell death, not only of the transduced cells, but of the neighboring cells (Fig. 2.6C). These data are consistent with the previous report that at high concentrations Erdr1 ceases to promote survival and instead induces cell death.[176] Thus, we were not able to generate a cell line that persisted after overexpression using either promoter and were unable to evaluate the effects of the overexpression of Erdr1 *in vivo*.

Induction of Apoptosis by Erdr1 Knockdown

Given the failure of sort-purified Erdr1 knockdown MEFs to expand *in vitro*, apoptosis in these lines was measured by flow cytometry using staining with Annexin-V 48 hours after transduction (Passage 1). At this time, there were only slight differences in percent apoptotic cells between Erdr1 knockdown MEFs (9.6% and 11.7%) and control shRNA MEFs (8.2%) (Fig. 2.7A). However, the ratio of Annexin-V cells in the knockdown vs. control MEFs increased over two further passages, reaching 1.7-fold and 2.8-fold (Fig. 2.7B). To determine whether Erdr1 knockdown would have the same effect in primary PMCs, these cells were transduced with the shRNA vectors and apoptosis was measured as before. Transduction efficiency in these cells was 48.1% for No Target shRNA, 43.4% for Erdr1 shRNA#1, and 61.0% for Erdr1 shRNA#2. As seen with the MEFs, PMCs transduced with the knock down shRNA had 3.1 and 2.5 -fold more apoptotic cells at passage 3 than the No Target shRNA line (Fig. 2.7C). Additionally, Erdr1 knockdown PMCs did not significantly expand (Fig. 2.7D). When measuring fold expansion over 15 days, No Target shRNA PMCs expanded 28.3 -fold while the two knockdown PMC cultures expanded a modest 1.17 -fold and 0.85 -fold. We next tested whether the increased apoptosis was capsase mediated, using the pan-caspase inhibitor Z-VAD-FMK. Fourteen days of treatment with mock inhibitor increased the percent apoptotic cells in Erdr1 knockdown PMCs to approximately 67%, while only 28% of the No Target shRNA transduced PMCs were apoptotic with mock inhibitor (Fig. 2.7E) ($p < 0.05$ for both knockdown PMCs vs. No Target shRNA PMCs). Z-VAD-FMK reduced No Target shRNA PMC apoptosis to that of the media alone group (12.9%), and partially reduced the percent of apoptotic Erdr1 knockdown

PMCs (to 27%, $p < 0.01$, and 37%, not significant, vs. mock inhibitor). Z-VAD-FMK also partially restored the ability of Erdr1 knockdown PMCs to expand *in vitro* (Fig. 2.7F).

V. Discussion

Metastasis is the most common cause of death for patients with cancer. This complex process requires that tumor cells migrate into and grow in distant tissue, which is dependent on cross-talk between the tissue stroma and the migrating tumor cells. However, designing strategies to disrupt this interaction have been difficult due to the lack of suitable stromal cell targets. Previously our group has shown that CCR5 signaling in stromal cells played a role in this process[170], and further investigation suggested that this role was early in the process of colonization, and partly involved MMP-9[171]. We hypothesized that other stromal genes involved in early colonization would be differentially expressed in the lungs of $CCR5^{-/-}$ mice after tumor injection, and designed the microarray experiment presented here to identify such potential targets. Our data identify Erdr1 as a pro-metastatic stromal cell survival factor regulated by CCR5.

We limited our study to genes differentially expressed at 6, 24, and 48 hours after tumor injection, and 5 of the 11 genes that met this criteria were differentially expressed more than two-fold at all three times. *Mid1* is the developmental gene implicated in X-linked Opitz G/BBB syndrome[197], and its expression has recently been reported to be predictive for colorectal carcinoma metastasis[198]. *Ccr9* is the gene for a chemokine receptor that multiple groups have linked to metastasis[199-201], as well as tumor growth [202, 203]. *Tna*, also known as tetranectin, was upregulated in $CCR5^{-/-}$ vs. WT mice in our study, but it has nevertheless been known for many years to be expressed in tumor stroma[204], and has more recently been associated with poor prognosis in a number of cancers[205-207] Also upregulated in $CCR5^{-/-}$ vs. WT lungs after tumor injection, *Clca3* is involved in immune responses induced by the Th2 cytokines IL-9 and IL-13, in both the lung and the colon[208,

209]. While any of these genes may be involved in early metastasis formation, which our pooled microarray data would support, we found their differential expression was not consistent between individual mice by semi-quatitative real time RT-PCR. This may be due to transient or context-dependent expression, or they may not be significantly linked to the role of CCR5 in metastasis.

On the other hand, we found Erdr1 to be consistently upregulated in WT lungs relative to $CCR5^{-/-}$ lungs during the first 48 hours after tumor injection. Furthermore, cultured PMCs from WT mice expressed more Erdr1 than $CCR5^{-/-}$ PMCs in every assay performed. This differential expression was seen not only in the $CD45^{+}$ fibrocyte population, which has been shown to respond to CCR5 stimulation[171], but also in $CD45^{-}$ PMCs. This could be explained by constitutive low-level expression of CCR5 on $CD45^{-}$ PMCs in combination with constitutive autocrine or paracrine chemokine release. Alternatively, CD45- PMCs could be "programmed" to express Erdr1 by CCR5 stimulation during differentiation from fibrocytes or mesenchymal stem cells, which also express CCR5[210]. The fact that Erdr1 is higher in WT PMCs even before contact with tumor cells suggests that its role in the lung is not necessarily limited to the period of early metastasis measured by our array, nor is it necessarily limited to metastasis. For instance, it may be involved in other processes dependent on CCR5 signaling in stromal cells, like experimentally induced pulmonary fibrosis.[45] In any case, these data are the first to demonstrate that Erdr1 expression is linked to ligand binding to CCR5.

Besides constitutive differential expression, our data also establish that stimulation of CCR5 on PMCs increases Erdr1 expression. The induction was largely confined to the $CD45^{+}$ fibrocyte population, as expected based on expression of CCR5. That fibrocytes

responded to CCR5 stimulation by expressing Erdr1 is particularly relevant given our previous data suggesting fibrocytes encounter and respond to CCR5 ligands in the lung during metastasis formation. Even given constitutive tissue stromal production of Erdr1, a local increase mediated by fibrocytes migrating into the new tumor site might then assist the formation of a tumor-supportive stroma.

To test the effect of knocking down Erdr1 in intravenously transferred stromal cells, we used MEFs rather than PMCs due to their greater ability to expand in culture, their phenotypic stability over several passages in culture, and the increased expression of Erdr1 in MEFs compared to PMCs. Initially, we confirmed the ability of MEFs to promote metastasis *in vivo*. We attempted to pursue similar studies using PMCs with the knockdown vectors described. However, Erdr1 is critical for the prevention of apoptosis in these cells and as a consequence, we were not able to generate a viable population of PMCs after transduction with vectors knocking down the expression of Erdr1 to do this analysis. A second approach, the generation of bone marrow chimeras using bone marrow cells transduced with lentiviruses expression shRNA for Erdr1, is complicated by the extremely slow turnover from bone marrow cells of fibrocytes. At this time, it is not clear when, if ever, all of the fibrocytes would convert from recipient to donor after transplantation.

When injected into mice prior to tumor injection, Erdr1 knockdown MEFs promoted fewer metastases than control shRNA MEFs in $CCR5^{-/-}$ but not WT mice. This is consistent with our previous finding that WT PMCs promoted metastasis in $CCR5^{-/-}$ mice, but $CCR5^{-/-}$ PMCs had no effect on metastasis in WT mice. Expression of Erdr1 in WT lungs may make the differences in Erdr1 expression between knockdown and control shRNA MEFs irrelevant to the process of metastasis. In $CCR5^{-/-}$ mice, however, the difference between lung Erdr1

content between groups was much larger, which may be critical to the observed effect on metastasis. We attribute some of the differences in metastasis promotion to the ability of Erdr1 to support survival of stromal cells. Knockdown of Erdr1 led to the gradual accumulation of apoptotic MEFs *in vitro*, and to a greater extent, of PMCs. The large increase in knockdown PMC apoptosis observed after incubation with a mock caspase inhibitor and 0.2% DMSO underscores the inability of these cells to cope with stress. Dormer *et al*. reported that murine erythroleukemia cells released Erdr1 when exposed to DMSO, as well as under other conditions of stress.[176] Therefore, the generation and release of Erdr1 may be an important anti-apoptotic response such stimuli.

Our data is not consistent with Erdr1 generating a proliferative advantage in stromal cells. Significant overexpression of Erdr1 by lentiviral vectors induced death of MEFs in culture. Thus, Erdr1 in both the Burkitt lymphoma cell line BL-70 and in primary fibroblasts appears to support both cellular survival and death depending on the degree of expression. It is possible that much lower expression of Erdr1 would enhance stromal cell proliferation although we were not able to demonstrate that.

One alternative explanation for our findings is that the knockdown of Erdr1 led to the injection of a greater number of apoptotic stromal cells compared to mock knockdown MEFs. We believe this is unlikely for two reasons. First, MEFs were injected 48 hours after transduction, at which time the percentage of apoptotic cells knockdown lines were still similar to the percentage in the No Target MEFs. Second, there was no difference in metastasis after injection of WT mice with knockdown MEFs, which would be expected if the small differences in apoptosis were inhibiting metastasis.

Rather, we propose that expression of Erdr1 allows MEFs to persist longer in the lungs after injection and promote the formation of tumor-supporting stroma. In the case of our previous study, induction of Erdr1 through stimulation of CCR5 on migrating fibrocytes would lead to locally higher concentration, thus supporting the formation of supportive stroma. In the case of natural *in vivo* metastasis a similar process may occur, whereby the colonization of tissues by metastatic nodules induces the migration of $CCR5^+$ fibrocytes and other $CCR5^+$ bone marrow-derived cells, which then produce *Erdr1* locally to support the tumor stroma. A recent study identifying Erdr1 as a survival factor for (HSCs)[183] is particularly relevant here, given that HSCs have also been implicated in the formation of pro-metastatic stroma[211].

The ongoing investigation in our laboratory of important aspects of Erdr1 function faces a number of challenges. The protein does not contain any of the known protein-protein domains or sequences critical for nuclear import or cellular secretion. A receptor for mediating the activity of Erdr1 has not been identified. The promoter is not known and the gene does not map currently to any part of the human genome although it has been shown to be expressed by multiple human cells. Array data suggests a critical role for Erdr1 during embryogenesis. Whether the anti-apoptotic role of Erdr1 is critical in this process is not clear at this time.

These findings identify Erdr1 as a cytokine important to the survival of peri-tumoral stromal cells. Furthermore, they establish an additional means by which inflammatory chemokines contribute to cancer progression. These data suggest that CCR5 may be a viable target for the prevention of tumor metastasis. Finally, our data suggest that this relatively unknown gene may be critical to understanding and manipulating tumor-stroma interactions.

VI. Experimental Procedures

Mice

C57BL/6J (WT) mice were purchased from Jackson Laboratories. $CCR5^{-/-}$ mice have been described previously.[212] All animals were housed in pathogen free conditions and animal experiments were conducted using protocols approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Cells

Isolation of pulmonary mesenchymal cells (PMCs) was performed as previously described[171]. In all experiments, PMCs were used within 2 passages. Murine embryonic fibroblasts (MEFs) were harvested from day E 13.5 embryos as described elsewhere [213] and were transduced following 2 and 5 *ex vivo* passages. All cell types were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. B16-F10 melanoma and A293T cells were purchased (American Type Culture Collection).

In Vivo Experiments

$4x10^5$ MEFs were transferred to mice by intravenous tail-vein injection. 48 hours later, 7.5×10^5 B16-F10 cells were injected intravenously. 14 days after tumor cell injection, mice were anesthesized by intraperitoneal injection of avertin, lungs were perfused with PBS, and the left lung was excised and fixed in Fekete's solution. Surface metastatic nodules were counted by an individual blinded to the experimental group.

Microarray Expression Analysis

Lungs from WT or CCR5 $^{-/-}$ mice were harvested at 6, 24, and 48 hours after intravenous injection of 7.5 x 10^5 B16-F10 melanoma cells (4 to 6 mice per group). RNA was isolated and reverse transcribed. The resulting cDNA from each group was pooled and hybridized to Affymetrix gene chips (6 chips total). Gene expression was analyzed using GeneSpring software. Confirmation of the overexpression of genes found from the microarray studies was performed using semi-quantitative RT-PCR. Primer sequences used are available in Supplementary Experimental Procedures.

Cytokine Stimulation

PMCs were cultured for 12 hours in serum-free DMEM, and then stimulated with 100 ng/mL of CCL4 (Peprotech). Serum-free media was replaced with 10% media 24 hours after stimulation; cells were harvested for RNA isolation 48 hours after stimulation.

RT-PCR and Western Blots

PMC or MEF RNA was isolated using the RNeasy Plus kit (Qiagen) and cDNA was reverse transcribed at 50℃ using Superscript III (Invitrogen) and oligo-dT. Remaining mRNA was degraded by RNAse H (Promega). Real Time PCR was performed using SYBR Green Master Mix, and Erdr1 expression was calculated relative to β-actin or SDHα . *Erdr1* specific primers were: Forward 5'-CCGCCGCGGTCAAGATGTATGT-3' and Reverse 5'-TTGACCACGGCGTCCGCTTCT A-3'. The *β-actin* specific primers were Forward 5'-TTCTTTGCAGCTCCTTCGTT-3' and Reverse 5'-GAGTCCTTCTGACCCATTC-3'. The

Sdhα specific primers were Forward 5'-GGAACACTCCAAAAACAGACCT-3' and Reverse 5'-CCACCACTGGGTATTGAGTAGAA-3'. Western blots were performed on whole cell lysates separated by 4-12% SDS-PAGE (Invitrogen), blocked with 3% bovine serum albumin, and probed with the Erdr1 specific rat antibody 8A12 (Ascenion). The rabbit anti-GAPDH antibody (sc-25778, Santa Cruz) was used as a loading control. HRP-conjugated goat anti-rat IgG (18-4818-82, Ebioscience) or donkey anti-rabbit IgG (18-8816-31, Ebioscience) were used as secondary antibodies, and blots were developed using an ECL kit (Amersham).

Cloning and Sequencing

Erdr1 was amplified from PMC and MEF cDNA using Accuprime GC Rich Polymerase (Invitrogen), with the primers (forward) 5'-GACCGTGCGGACTTAAGATGG-3' and (reverse) 5'-TTATTGAGGGGGGGCATTTCTGTA-3', and 40 cycles of 95 ^{o}C for 30 sec, 60 ^{o}C for 30 sec, and 72 ^{o}C for 2 min (adapted from [175]). PCR products were cloned into pCR-BluntII-TOPO (Invitrogen) and transformed into TOP10 cells (Invitrogen). Kanamycin resistant clones were screened for inserts by EcoRI digestion (New England Biolabs) and sequenced by the UNC Genome Analysis Facility using the primers provided by the manufacturer. For expression by lentiviral vectors, *Erdr1* cDNA was cloned into pLenti7.3 (Invitrogen). Another pLenti7.3 construct with an EF-1α promoter was obtained by a restriction cloning strategy, involving the removal of the CMV promoter from pLenti7.3-Erdr1, the removal of the EF-1α promoter from pEF-DEST (Invitrogen), and the ligation of the EF-1α promoter into the promoterless pLenti7.3-Erdr1 plasmid. Lentiviral vectors were packaged in 293T cells according to the manufacturer's instructions.

Flow cytometry and cell sorting

For identification of PMC populations, cells were harvested by trypsinization and resuspended in PBS with 2% FCS, then incubated with anti-Thy1.2-PECy7 (eBioscience) and anti-CD45-eFluor450 (eBioscience) antibodies for 15 min. at room temperature. $CD45^+$ cells were sorted by the UNC Flow Cytometry Facility. For identification of apoptotic cells, PMCs or MEFs were resuspended in Annexin-V binding buffer and stained with propidium iodide (eBioscience) and Annexin-V- APC (eBioscience) for 15 min at room temperature. Fluorescence was measured on the MACSQuant Analyzer (Miltenyi), and further analyzed using Summit software (Beckman Coulter).

Generation and use of shRNA retroviral vectors

One control shRNA sequence designed not to target any known gene, and two shRNA sequences targeting Erdr1, based on siRNA sequences from Dharmacon (No Target shRNA: D-001810-01-05, Erdr1 shRNA #1: J-053706-09, Erdr1 shRNA #2: J-053706-11) that were validated for knockdown efficiency, were cloned into pHSPG vector [214, 215] driven by an histone H1 promoter. The shRNA sequences were confirmed by the UNC Genome Analysis facility. The pHSPG shRNA constructs were co-transfected with plasmids containing the VSV-G and gag/pol genes into A293T cells by calcium phosphate transfection as described [215]. Supernatant containing recombinant virus was harvested at various time points between 36 and 72 hours post-transfection and passed through a 0.45 micron filter. The packaged shRNA virus was concentrated by centrifuging at 24,000 rpm for 3 hours at 4^o C and followed by re-suspension in PBS. Viral titer was determined by transducing NIH 3T3 cells and assaying for GFP expression by flow cytometry 48 hours after transduction. PMCs

or MEFS were transduced in 6-well plates by adding polybrene (4 μg/mL) and virus (MOI=5) to the media and centrifuging the plates at 1000 X g for 1.5 hours. Media was replaced with fresh media after 24 hours, and transduction efficiency (% GFP^+) was measured by flow cytometry 48 hours post-transduction.

Statistics

Unless otherwise stated, data are presented as the mean of measurements taken from three or more separate experiments. Statistical error for these means is presented as ± 1 SEM. *p* values were determined by Student's T-Test, and values ≤ 0.05 were considered significant.

Acknowledgements

We thank Peter Dormer for initial assistance obtaining antibody for Western blots, and Deborah Taxman for advice on constructing shRNA vectors. This work was supported by NIH Grant P50CA058223 (JSS) and ACS Grant RSG 118750 (HVD). RLM is a member of the Medical Scientist Training Program T32 GM008719.

VII. Figures

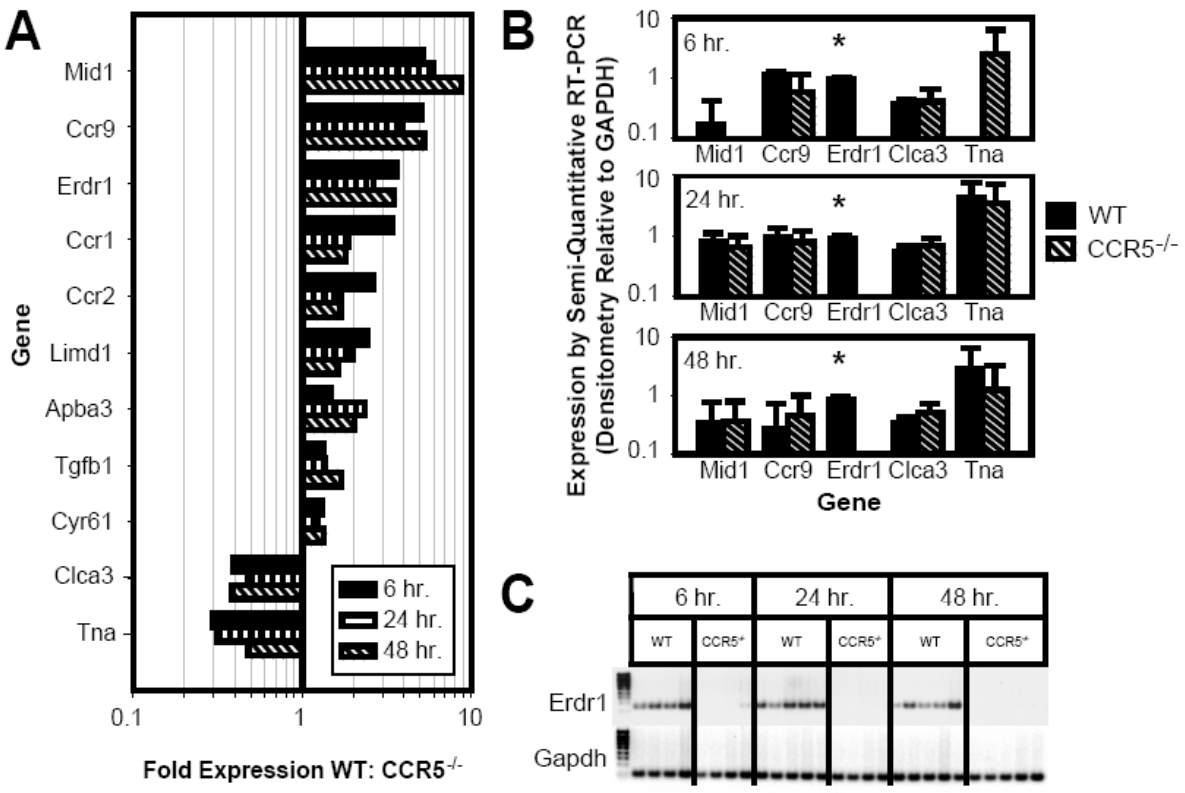


Figure 2.1 Erdr1 is upregulated in WT vs. CCR5$^{-/-}$ lungs after IV tumor injection. Lungs were harvested 6, 24, and 48 hr after IV B16-F10 injection, and RNA was immediately isolated for Affymetrix array and RT- PCR. **A.** Genes differentially expressed in WT vs. CCR5 $^{-/-}$ lungs at all three time points, measured by Affymetrix array. RNA was pooled from the lungs of mice in each group at each time point. **B.** Semi-quantitaive RT-PCR using primers for genes differentially expressed by more than 2-fold on the Affymetrix array. Numbers represent averages of densitometry values for RT-PCR of lung RNA from the individual mice used in the Affymetrix array. Error bars represent $^{\pm}$ SEM. * $p < 0.05$ for WT vs. CCR5$^{-/-}$ All other differences were not statistically significant. **C.** Semi-quantitative RT-PCR for Erdr1, showing consistent differential expression for all mice at all time points.

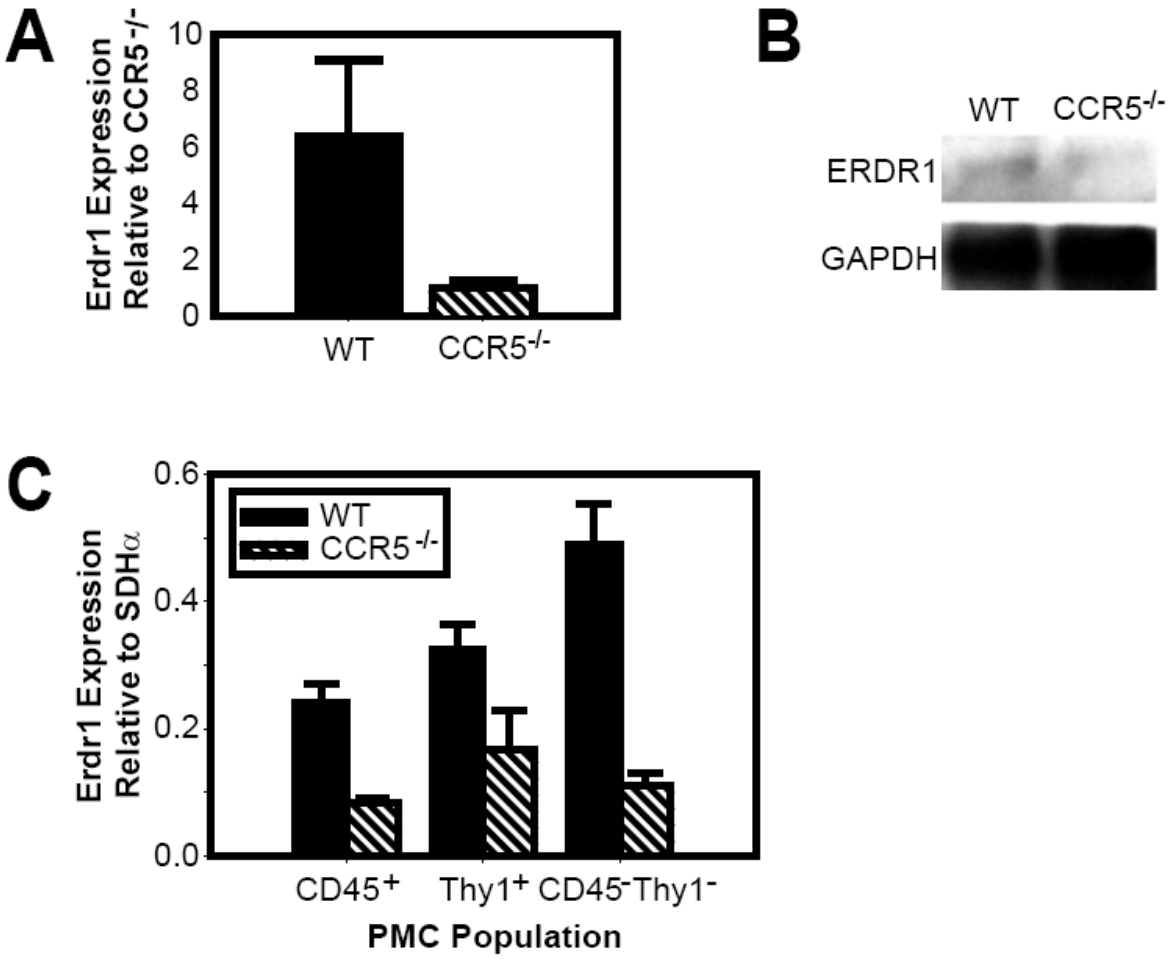


Figure 2.2 Erdr1 is upregulated in WT vs. CCR5$^{-/-}$ PMCs in culture.

PMCs were cultured from perfused WT or CCR5-/- lungs for 2-3 passages, and then harvested for RNA and protein analysis. **A.** Erdr1 expression relative to CCR5$^{-/-}$ PMCs by real time RT-PCR. Erdr1 expression was normalized to β-actin. Results averaged from 3 separate concurrent cultures of WT and CCR5$^{-/-}$ PMCs. **B.** Erdr1 protein expression in WT and CCR5$^{-/-}$ PMCs by Western blot. **C.** Differential Erdr1 expression in WT and CCR5$^{-/-}$ PMC populations by real time RT-PCR, relative to SDHα. Error bars represent $^{\pm}$ SEM. * $p < 0.005$ for WT vs. CCR5$^{-/-}$ # $p < 0.05$ for WT vs. CCR5$^{-/-}$ ‡ $p < 0.05$ for WT CD45^{-}Thy1^{-} vs. WT CD45^{+}.

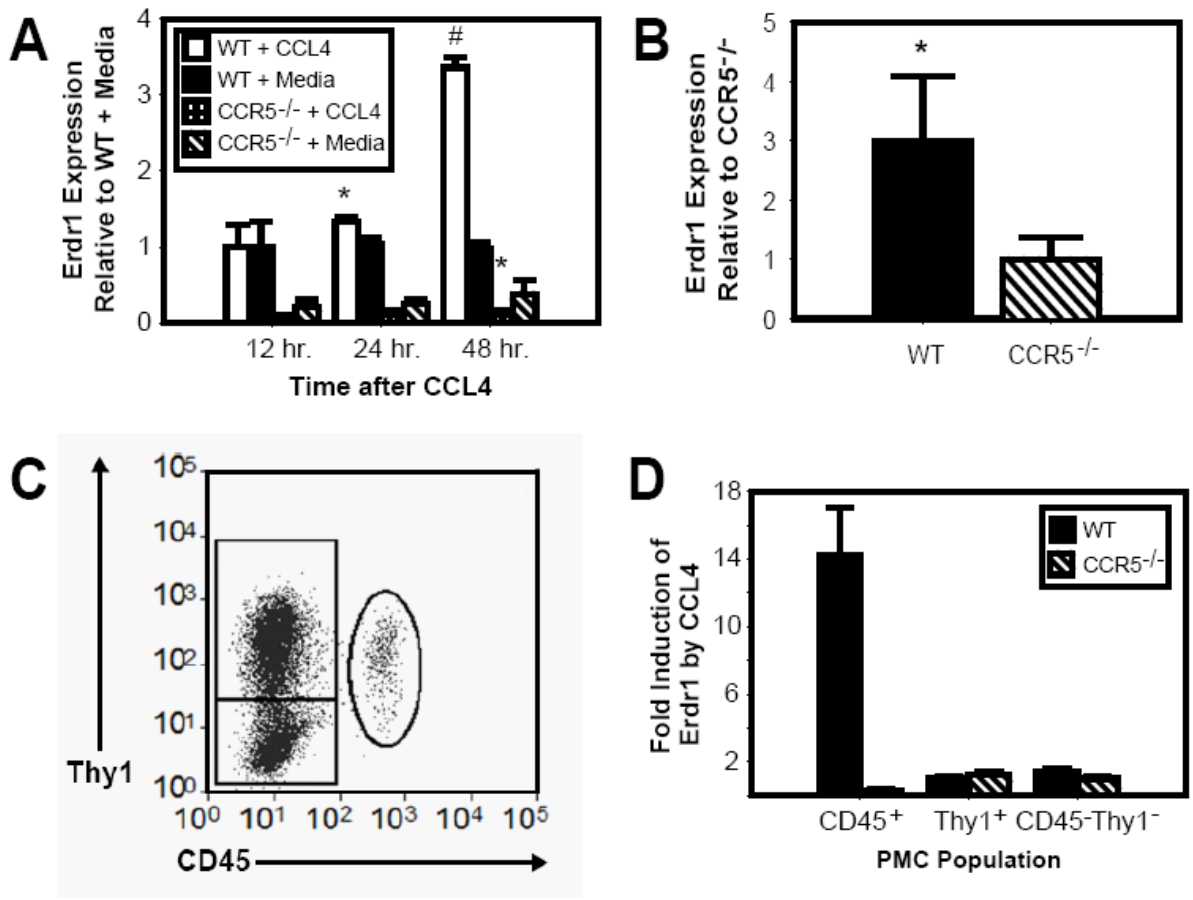


Figure 2.3 Stimulation of CCR5 on PMCs induces expression of Erdr1.

WT and CCR5$^{-/-}$ PMCs were stimulated with CCL4 or media alone and harvested after the given intervals for RNA analysis. A. Erdr1 expression at 0, 24, 48 hr after addition of CCL4 by real time RT-PCR. * $p < 0.05$ for CCL4 vs. media # $p < 0.005$ for CCL4 vs. media. B. Fold induction of Erdr1 at 48 hours after CCL4 stimulation. Numbers represent the mean of 3 separate experiments. * $p < 0.05$ for WT vs. CCR5$^{-/-}$. C. Flow cytometric analysis of PMC populations sorted by CD45 and Thy1 expression for Erdr1 real time RT-PCR. D. Fold induction of Erdr1 in sorted PMC populations. PMCs were sorted 48 hours after stimulation with CCL4. ‡ $p < 0.01$ Error bars represent ± 1 SEM.

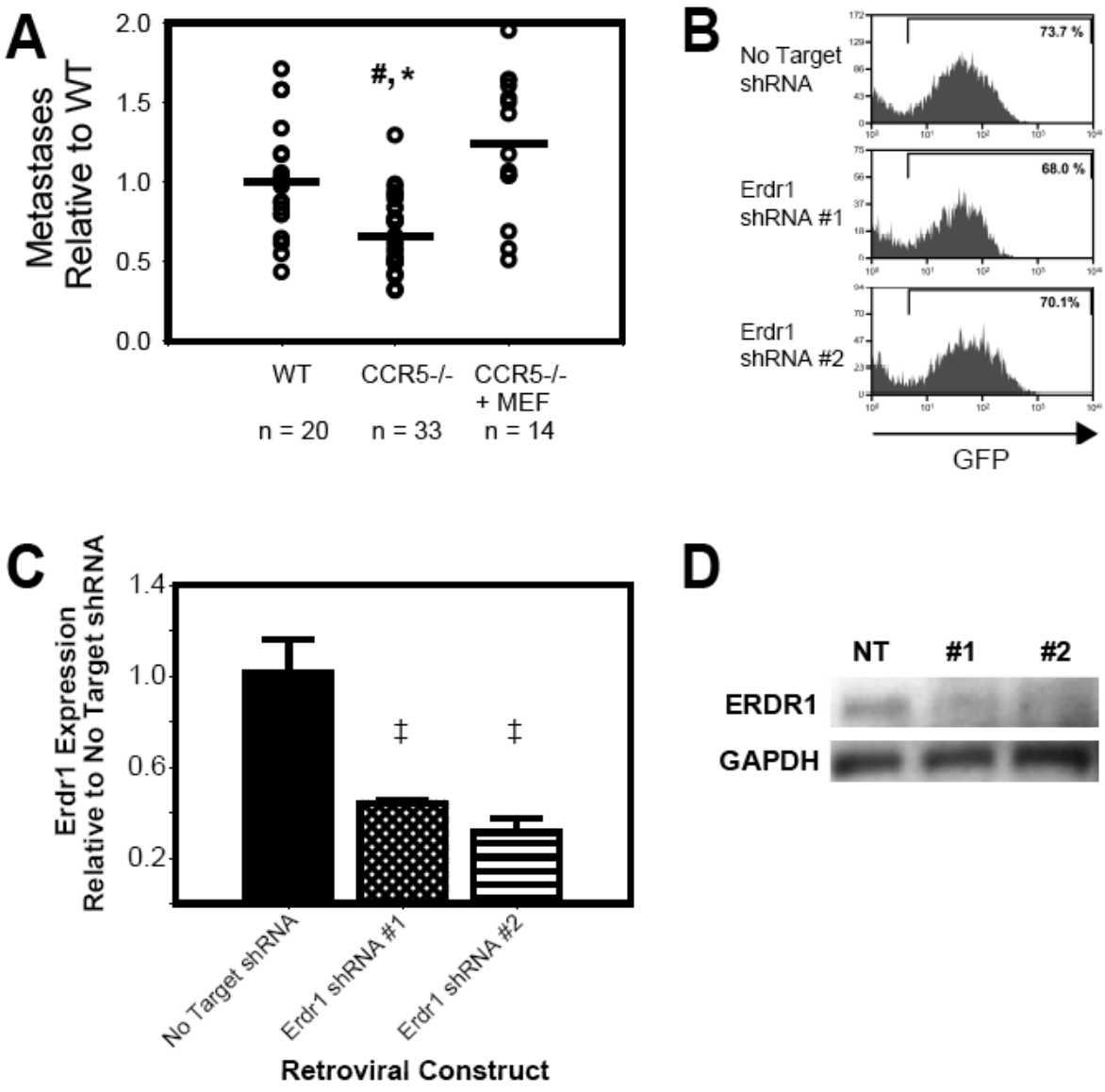


Figure 2.4 MEFs promote metastasis and shRNA reduces Erdr1 expression in MEFs. **A.** Metastasis formation in WT and CCR5$^{-/-}$ mice, and CCR5$^{-/-}$ mice were injected with MEFs prior to B16-F10 injection, relative to the mean number of metastases in WT mice. * $p < 0.001$ for WT vs. CCR5$^{-/-}$ # $p < 0.001$ for CCR5$^{-/-}$ vs. CCR5$^{-/-}$ + MEFs. Metastasis in WT vs. CCR5$^{-/-}$ + MEFs was not statistically significant ($p = 0.09$). **B.** Percent GFP^{+} cells in MEF cultures transduced with shRNA retroviral constructs 48 hours after transduction. **C.** Erdr1 expression in MEFs cultures transduced with shRNA retroviral constructs by real time

RT-PCR 48 hours after transduction. Error bars represent $^{\pm}$ 1 SEM. ‡ $p < 0.05$ vs. No Target shRNA. **D.** Erdr1 protein expression in MEFs cultures transduced with shRNA retroviral constructs by Western blot.

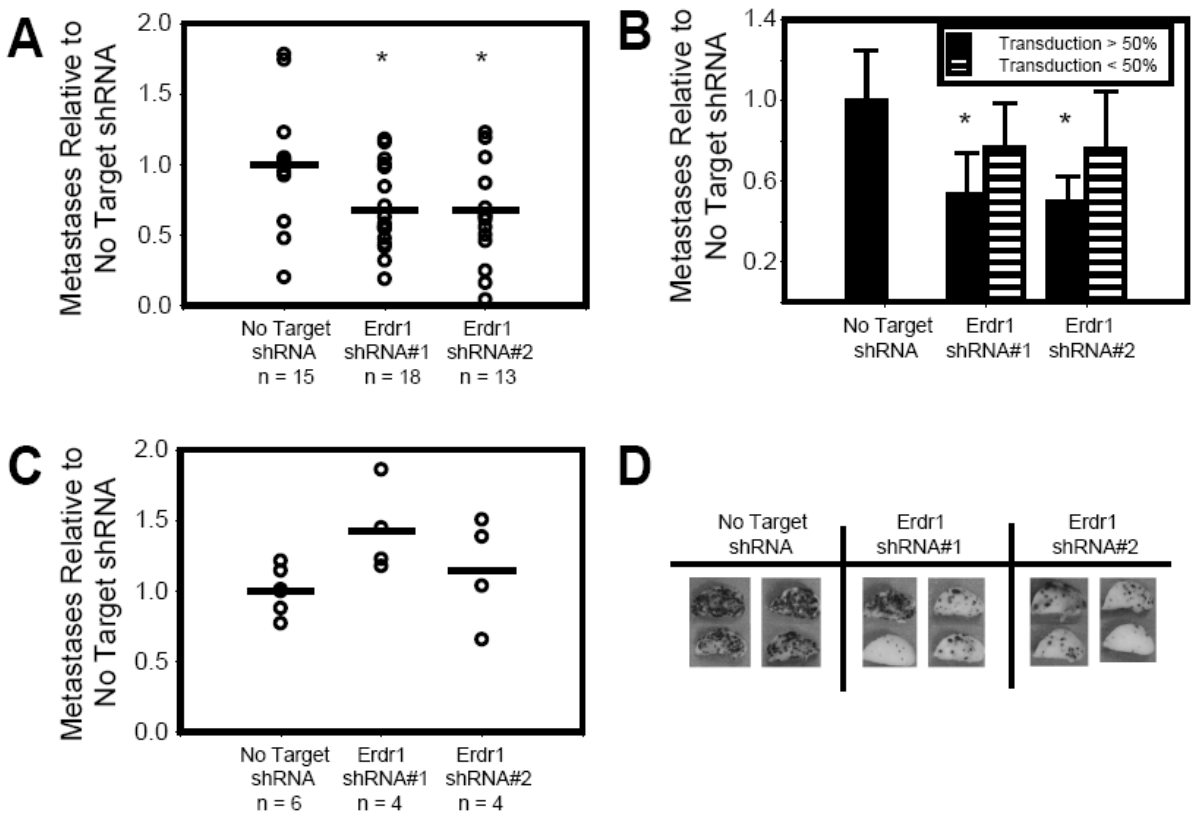


Figure 2.5 Erdr1 knockdown MEFs promote fewer metastases in CCR5 $^{-/-}$, but not WT mice.

MEFs were transduced with Erdr1 targeting or No Target shRNA expressing retroviral vectors, and injected into mice 48 – 72 hours after transduction. B16-F10 cells were injected 48 hours later, and mice were harvested after 14 days for enumeration of metastases in the left lung. **A.** Metastases in lungs of CCR5$^{-/-}$ mice injected with shRNA transduced MEFs prior to B16-F10 injection, relative to mean number of metastases in the No Target shRNA group for each experiment. Results pooled from 3 separate experiments. * $p < 0.05$ vs. No Target shRNA. **B.** Metastases in lungs of CCR5$^{-/-}$ mice injected with shRNA transduced MEFs prior to B16-F10 injection, analysed by transduction efficiency of the injected MEFs. Expressed as relative to mean number of metastases in the No Target shRNA group for each experiment. Results pooled from 3 separate experiments. * $p < 0.05$ vs. No Target shRNA. Mean relative metastasis in < 50% transduction groups were not statistically different from

No Target shRNA ($p = 0.10$ and $p = 0.15$). **C.** Metastases in lungs of WT mice injected with shRNA transduced MEFs prior to B16-F10 injection, relative to mean number of metastases in the No Target shRNA group. **D.** Lungs of $CCR5^{-/-}$ mice injected with shRNA transduced MEFs prior to B16-F10 injection, from an experiment with > 50% transduction.

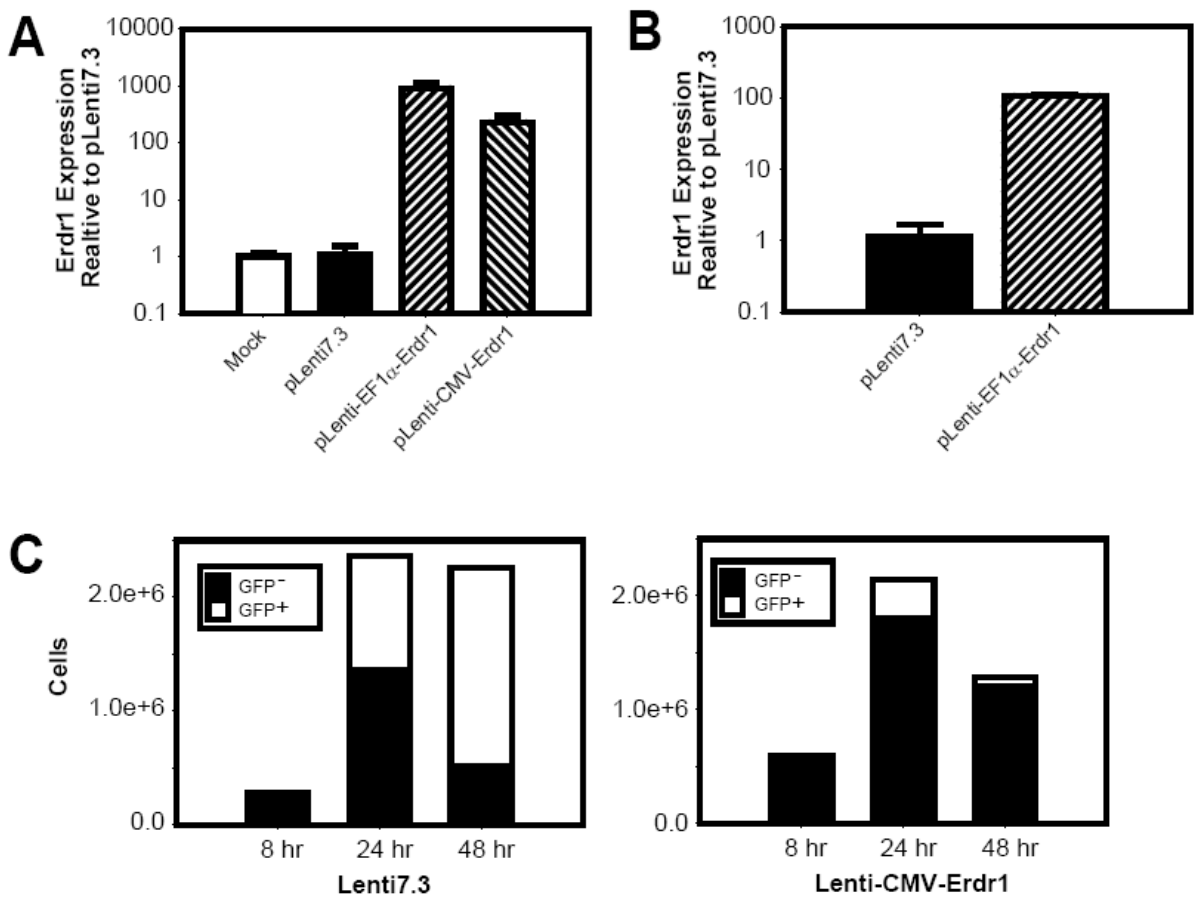


Figure 2.6 Erdr1 Overexpression leads to rapid cell death *in vitro*.

A. Erdr1 expression in transfected A293Ts, relative to A293T + empty plasmid. **B.** Erdr1 expression in lentivirus transduced PMCs, relative to empty vector. **C.** Enumeration of MEFs at 8, 24, and 48 hours post-transduction. GFP expression indicates transduced cell.

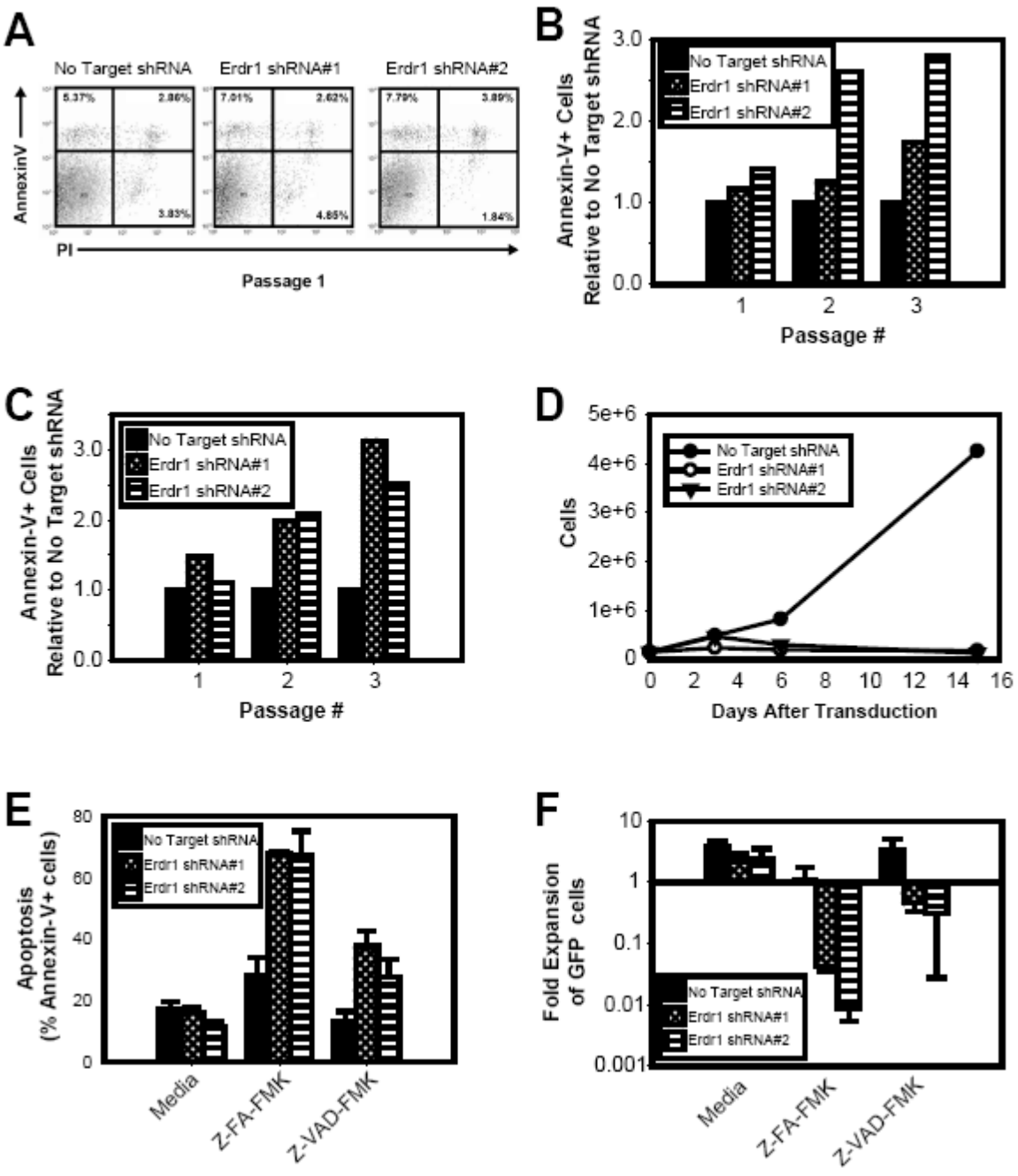


Figure 2.7 Erdr1 knockdown induces apoptosis in MEFs and PMCs.

A. Apoptosis in Erdr1 knockdown MEFs 48 hr after transduction (passage 1) by staining with Annexin-V and PI. **B.** Apoptosis in Erdr1 knockdown MEFs over 3 passages after transduction, expressed as fold difference in % Annexin-V^+ cells relative to No Target shRNA. **C.** Apoptosis in Erdr1 knockdown PMCs over 3 passages after transduction,

expressed as fold difference in % Annexin-V^+ cells relative to No Target shRNA. **D.** Cell number in Erdr1 knockdown PMC cultures over 3 passages (15 days) after transduction. **E.** Effect of caspase inhibition on Erdr1 knockdown PMC apoptosis. PMCs were transduced with Erdr1 knockdown or No Target shRNA, and treated with media, a pan caspase inhibitor (Z-VAD-FMK), or a mock inhibitor (Z-FA-FMK) every 3 days, and analyzed after 14 days. **F.** Effect of caspase inhibition on Erdr1 knockdown PMC fold expansion.

VIII. Supplementary Figures

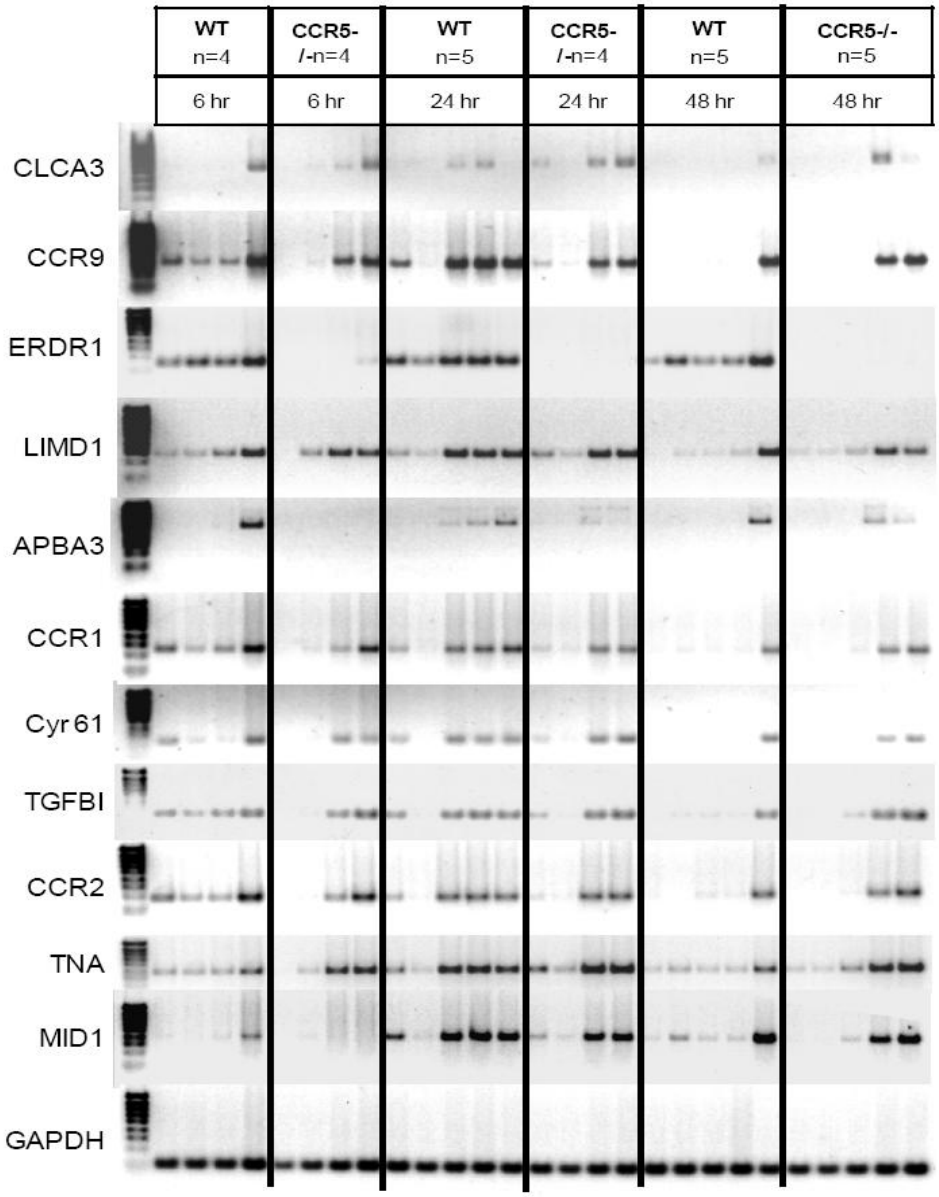


Figure 2.S1 Semi-quantitative RT-PCR for genes differentially regulated at all 3 time points by Affymetrix array.

Figure 2.S2 Sequence alignment for the consensus Erdr1 sequence from PMC cDNA with published sequences.

The consensus sequence was derived from more than 20 clones, and from different PMC cultures.

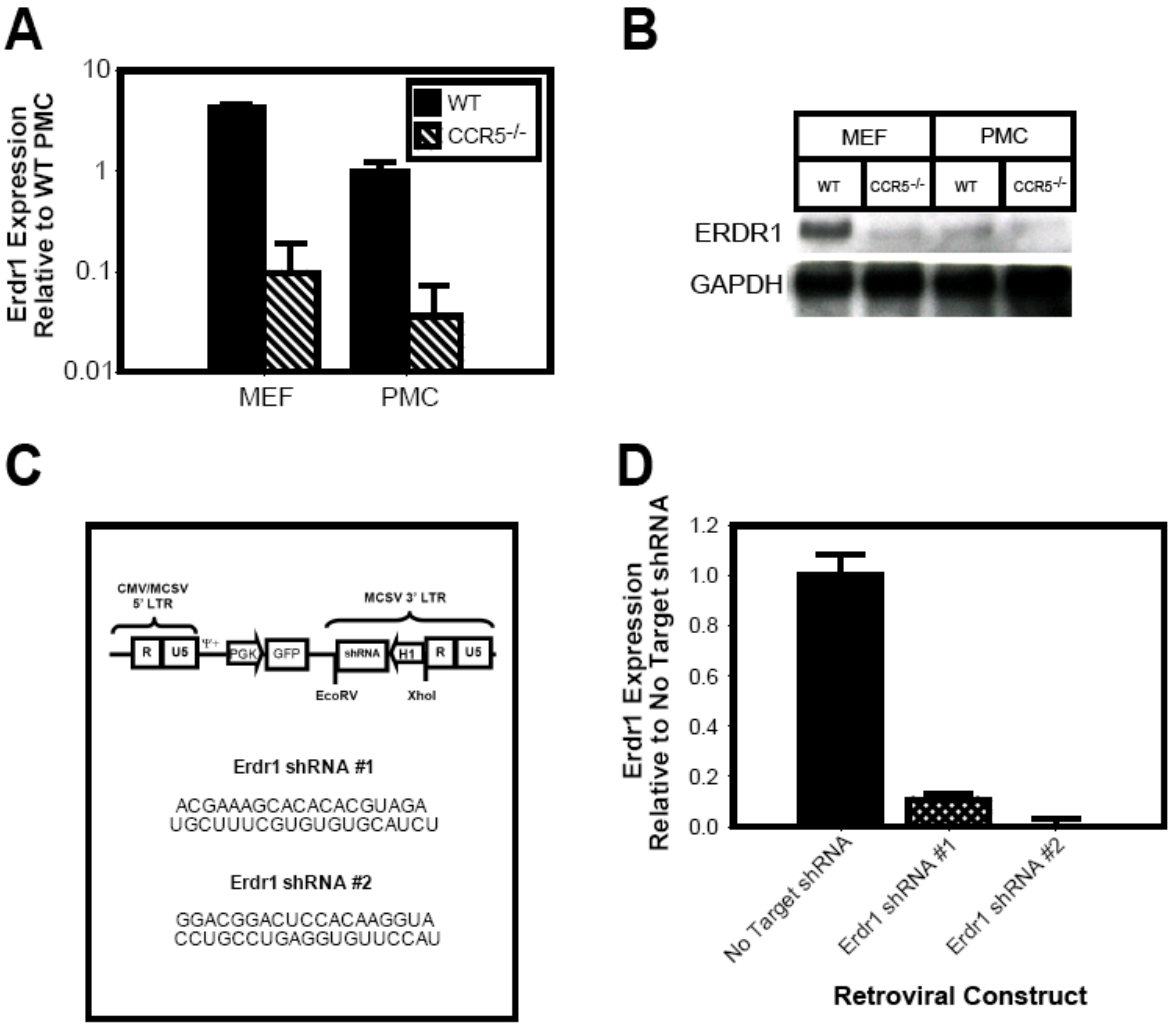


Figure 2.S3 Erdr1 expression in MEFs.

A. Erdr1 expression in WT and CCR5$^{-/-}$ MEFs and PMCs by real time RT-PCR, expressed relative to WT PMCs. All groups were statistically significantly different from each other. **B**. Erdr1 protein expression in WT and CCR5-/- MEFs and PMCs by Western blot. **C**. Map of retroviral construct used for shRNA knockdown, and shRNA sequences. **D**. Erdr1 expression in sort-purified MEFs transduced with shRNA retroviral constructs and sorted 48 hours after transduction. Real time RT-PCR results expressed relative to No Target shRNA. Error bars represent ± 1 SEM * $p < 0.01$

Chapter 3
Molecular Characterization of Erythroid Differentiation Regulator 1

I. Abstract

Erythroid Differentiation Regulator 1 (Erdr1) has been shown to induce hemoglobin synthesis, to promote the proliferation and prevent apoptosis of cell lines, and to promote hematopoietic stem cell activity. We have recently found that Erdr1 promotes metastasis as a stromal cell survival factor, and is induced upon stimulation of the chemokine receptor CCR5. Though evidence concerning the functions of Erdr1 is building, many questions about its molecular biology remain, particularly with regard to its existence in humans. This chapter will discuss the molecular characterization of Erdr1. We have found Erdr1 expression in multiple murine and human cell lines and primary cells. The description of the initial isolation of Erdr1 refers to high variability in the 5' end of RNA transcripts. The sequence we obtained from pulmonary mesenchymal cell cDNA differed from the published sequences in that region, as previously described. We also found multiple products by 5' RACE without start sites, consistent with other observations that many *Erdr1* mRNA sequences do not contain open reading frames (ORFs). Using a combinatorial primer approach, we show that an identical ORF was expressed in the human Raji cell line, as well as in primary nurse-like cells from CLL patients. These data demonstrate that the ORF found in PMCs expresses a protein and is conserved in both mice and humans.

II. Introduction

Evidence is growing that Erdr1 plays a role in multiple biological processes such as induction of hemoglobin in erythroleukemia cells[175], survival of Burkitt's lymphoma cells and erythroid and granunlocyte/monocyte progenitors[176], enhancement of hematopoietic stem cell repopulation[183], and promotion of metastasis by stromal cells (see Chapter 2). It has also been associated with many other processes through expression array studies. However, there are still currently many questions about this gene that must be addressed before these findings can be of clinical benefit. For example, though its expression has been reported in human cells[175, 176], there is no publically available sequence for the human homolog, and BLAST searches for human genes result in no homology. In mice there are no other genes with significant homology to *Erdr1*, and there are no conserved domains in its sequence, hampering hypothesis generation regarding its biochemical mechanisms of action. Its chromosomal location in mice is also not definitively known, although there is some evidence placing it on the distal end of the X chromosome[193].

There is also some discrepancy in the transcript sequence. Erdr1 was first isolated from a WEHI-3B cDNA library expressed in COS-1 cells, and screened for the ability to induce hemoglobin synthesis. In one clone with this activity, a 534 bp open reading frame (ORF) was identified. Using this sequence as a probe, a larger sequence was identified, which was then used to study *Erdr1* RNA species by Northern blot. Multiple bands were found which, when sequenced, had a highly variable region of repetitive sequences upstream from the originally identified start site and a short 5' consensus region. None contained ORFs. However, when that consensus sequence was used as a forward primer for RT-PCR

of WEHI-3B and mouse spleen cDNA, a transcript was amplified that contained an ORF with the same stop site as the 534 bp ORF, but with a start site in the 5' consensus sequence (AJ539223). In this larger ORF (630 bp) the variable region was absent, suggesting that it was an intron. The 630 bp ORF was also identified in cDNA from human PBMCs, and was reported to have an identical sequence.[175]

We created a clone of *Erdr1* from PMCs for the generation of protein and for mutational analysis of function and protein-protein interactions,. While Dormer *et al*. did report expression in humans, we wanted to confirm this finding, given the absence of the sequence in the human public databases and the improbability of 100% identity between human and mouse sequences. Here we show expression of Erdr1 in multiple mouse and human cell types, describe the methods by which Erdr1 was cloned from PMCs, analyze mouse transcripts by rapid amplification of cDNA ends (RACE), and provide evidence for expression of this ORF by a human cell line and primary human cells.

III. Results and Discussion

Cloning of Mouse *Erdr1*

We first determined Erdr1 expression using a primer pair specific for a 150 bp segment near the 3' end of the gene. These primers have been used to validate differential expression on a microarray with semi-quantitative RT-PCR, and were later used for real time RT-PCR (see Chapter 2). This segment of *Erdr1* cDNA was easily detectible in mouse PMCs, murine embryonic fibroblasts (MEFs), WEHI-3B cells, as well as B16-F10 melanoma cells (Fig. 3.1A). The last of these is particularly interesting given our data that stromal Erdr1 expression promotes metastasis. We had found no difference in the ability of Erdr1 knockdown cells to promote B16-F10 proliferation *in vitro* (not shown), and this may be partly due to expression by the tumor cells themselves.

We cloned Erdr1 from PMC cDNA to determine the transcript relevant to our metastasis findings. Initially, we did not detect any PCR product using a forward primer beginning at the start site of the 534 bp ORF. When the forward primer used by Dormer *et al.*, in the 5' consensus region, was substituted, we detected multiple products of differing lengths (Fig. 3.1B). When these were sequenced, multiple gaps were noted compared to the published sequences and no ORFs were present. A number of modifications were initially made to optimize the PCR conditions that were not successful, including changing the concentration of Mg^{+2}, and the PCR reaction conditions.

The high GC content of the gene (68.4%) and a region near the 3' end with a high potential for forming duplexes likely contributed to the difficulties encountered with RT-

PCR analysis. A possible problem with such a sequence is the generation of RNA or cDNA secondary structures that would hamper reverse transcription, primer annealing, or elongation. Thus, we used a high temperature reverse transcriptase with activity at 55^{o}C, in order denature RNA structures. Additionally we used a specialized Taq polymerase system designed to reduce the strength of the hydrogen bonding in G-C rich areas and optimized the annealing temperature, elongation time, and the concentration of primers and Mg^{+2}. This approach yielded Erdr1 PCR products (Fig. 3.1C) and when these were cloned and sequenced, we found a 534 bp ORF sequence that was similar to the original sequence of *Erdr1* obtained from WEHI-3B cells by Dormer *et al*. This sequence is provided in Chapter 2.

RACE to Evaluate Start and Stop Sites in *Erdr1*

As indicated the 534 bp sequence had an ORF that corresponded to the published sequence from the mammary tumor cell line, but was not identical to the sequence that had been characterized from WEHI cells. A more definitive method to evaluate the 5' and 3' start and stop sites in genes is RACE-PCR. For 5' RACE, the 5' methylguanosine caps of processed transcripts are replaced with an adapter sequence, and two rounds of nested PCR are performed using gene specific and adapter specific primers. For 3' RACE, gene specific primers are used in combination with poly-T primers that bind the 3' tail. This allows specific amplification of targets without prior knowledge of the entire transcript sequence.[216] We used two different pairs of reverse primers for 5' RACE, and two different pairs of forward primers for 3' RACE, as shown in Fig. 3.2A. The first round of replication yielded 3' RACE products of the expected sizes (Fig. 3.2B), indicating that the 3' end of the PMC *Erdr1* transcripts agreed with our sequence.

The 5' RACE products were varied after the first round, which might be due to non-specific primer binding. The second round of nested PCR also yielded multiple products for the 5' RACE, none of which were the expected length (Fig. 3.2C). Even more bands were detected when this was repeated. They were identical between 4 replicate reactions, though some differences were seen between samples 1 and 2, which were separately ligated to the 5' adapter (Fig. 3.2D). The specificity of these reactions did not improve with differing annealing temperatures or Mg^{2+} concentrations (not shown). These products were pooled and the largest band (~350 bp) was cloned and sequenced.

As shown in Fig. 3.3, there were multiple different sequences, all of which aligned with *Erdr1* at the 3' end, but had varying homology toward the 5' end. The 5' region of homology shared by every clone represents the 5' adapter sequence, and is thus an artifact. From the sequence alignment, these appear to result from truncations in the expected *Erdr1* sequence, perhaps from targeted RNA degradation, or shear stress on 3 dimensional RNA structure. We do not think this a consequence of the quality of the RNA, since the 3' RACE for *Erdr1* and the 5' RACE for β-actin both produced products of the expected sizes. Thus, we were unable to generate a 5' RACE product that contained the putative start site in *Erdr1*.

This result appears to be specific to the 5' end of *Erdr1*, confirming the RNA variability described by Dormer *et al*. They did not find *Erdr1* transcripts with ORFs by Northern blot, indicating an abundance of pre-spliced or partially degraded *Erdr1* mRNA. The 5' variability and repeated sequences could make RACE PCR amplification of this region difficult. Furthermore, if *Erdr1* transcripts are not fully processed until translation is required, as proposed by Dormer *et al*., there may be few at any given time with 5' methylguanosine caps, which are added to mature transcripts.[217] Thus, the adapter

sequence used for 5' RACE might instead be ligated to the free ends of degraded transcripts, yielding incomplete sequences after PCR.

These factors could explain why databases derived from high throughput sequencing projects contain very few *Erdr1* related sequences in the mouse, and none in the human. Indeed, we initially had difficulty amplifying the full-length gene with fidelity before optimizing both the reverse transcription and PCR reaction conditions. Therefore, approaches specifically designed to detect *Erdr1* sequences might be more successful than high throughput methods.

Cloning of Human Erdr1

We next designed a protocol to determine the sequence of human Erdr1. The 150 bp segment of *Erdr1* was also present in human PBMC-derived fibrocytes (Fig. 3.4A) as well as PBMCs from healthy donors and CLL patients (Fig. 3.4B). The identification of Erdr1 expression in human fibrocytes suggests that its induction in fibrocytes though CCR5 signaling may be relevant in human processes as well. We also found Erdr1 protein production by Western blot. The same antibody that binds Erdr1 in MEF lysates also binds a protein of the same size in lysate from the PBMCs from 1 of 2 healthy donors, as well as in a lysate from healthy human bone marrow (Fig. 3.4C). Similarly, Dormer *et al*. also found variability in the expression of Erdr1 protein between the PBMCs of healthy donors.[175]

For the PBMCs, the full length *Erdr1* ORF was also identified (Fig. 3.4B). Dormer *et al*. reported an identical sequence for human and mouse *Erdr1*, and we also obtained an identical sequence (not shown) from the CLL patient PBMCs. One obvious concern with

finding that the human and mouse homologs of *Erdr1* were identical is that 100% interspecies sequence identity is uncommon. The average sequence identity between mouse and human genes is 85%, with a range of 36% to 100%.[218] The average is the same for amino acid and nucleotide identity, but even for highly conserved genes, 100% nucleotide identity is rare. This is because mutations in the third nucleotide of each codon often have no effect on protein function, due to the redundancy of the genetic code. Accordingly, a survey of 1196 human-mouse homologous gene pairs found 27 with identical protein sequences, but only 2 with identical nucleotide sequences.[218] These 2 were not identified, but the most highly conserved genes tend to encode critical and structurally finely-tuned proteins, such as cytoskeletal, DNA-binding, or RNA-binding proteins.[218, 219]

Given the potential role of Erdr1 in many biological processes, and the detrimental effect of Erdr1 knockdown in fibroblasts, there may be considerable selective pressure against genetic drift in this gene. Supporting this hypothesis is an expressed sequence tag (EST) isolated from the little skate (EE990695.1) with 93% sequence identity to a 3' 272 bp region of mouse *Erdr1*. However, due to the scarcity of genes with 100% nucleotide identity between mice and humans, we were concerned that the human sequence reflected contamination from murine cDNA. To increase the possibility of detecting sequence differences, we designed 12 forward and 9 reverse primers based on the 534 bp ORF (Fig. 3.5A, Table 3.1), which could be combined into 64 unique PCRs. To reduce the risk of mouse contamination, this experiment was performed in a separate room of the Lineberger Comprehensive Cancer Center which has not had exposure to mice with new unopened reagents. Thus, this approach greatly reduced the likelihood of failing to amplify the human

sequence due to lack of primer homology and significantly limited the concerns that the product we generated was due to contamination from murine sequences.

The human Burkitt's lymphoma cell line Raji was used as a source of RNA, because the Burkitt's cell line BL-70 was reported by Dormer *et al*. to be Erdr1^{+} by Western blot[176], and because much higher amounts of RNA could be produced from Raji cells than from primary human cells. As shown in Fig. 3.5B, 36 of the 64 PCRs had products and 23 of those were near the predicted size (arrows). This was highly suggestive of close homology between mouse and human *Erdr1*. 9 of these PCRs were cloned and sequenced, and 2 of these contained sequences identical to mouse *Erdr1* (Fig. 3.6). All other clones analyzed contained unrelated human genes, and none contained mouse genes. One of the two clones was the full length 534 bp ORF, and the other started at position 442 (on AJ539223) and ended at the stop codon. Each clone had a 1 bp difference from the mouse sequence conserved in the forward and reverse sequencing reactions, but these were at different positions, and so were attributed to sequencing or PCR error.

Human nurse-like cells express an identical *Erdr1* sequence to mouse cells

To further confirm these findings, we next sequenced *Erdr1* transcripts from primary human cells. Nurse-like cells (NLCs) are stromal cells that are often found in the peripheral blood of CLL patients, and they support the growth of CLL cells *in vitro* via CXCL12.[220] As they are circulating cells that express stromal cell markers and support tumor growth, they may be phenotypically related to fibrocytes. Our data indicate that fibrocytes express Erdr1,

and that PBMCs from CLL patients express Erdr1 (though not more than PBMCs from healthy donors), so we determined if NLCs expressed Erdr1 .

As shown in Fig. 3.7, 3 different primer pairs led to the products of the predicted sizes using NLC cDNA. Thus it is highly likely that these cells express Erdr1. Two of these products that together spanned the ORF were cloned and sequenced. As with the Raji cells, the consensus sequence was identical to the 534 bp ORF of mouse Erdr1 (Fig. 3.8). Whether Erdr1 expression by NLCs contributes to their support of CLL cells is the subject of ongoing studies.

From these data, we conclude that mouse and human cells express Erdr1 transcripts containing a 534 bp ORF. It is difficult to know whether this ORF represents the final transcript, but forced expression of it causes rapid death in MEFs (see Chapter 2). Furthermore, our data support the previous report that human *Erdr1* is identical to mouse *Erdr1*.

IV. Experimental Procedures

Mice

C57BL/6J (WT) mice were purchased from Jackson Laboratories. All animals were housed in pathogen free conditions and animal experiments were conducted using protocols approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Cells

Isolation of pulmonary mesenchymal cells (PMCs) was performed as previously described[171]. In brief, single cell suspensions of pulmonary cells were formed by digesting PBS-perfused lungs with collagenase (Sigma) and DNAse I type II (Sigma), and then lysing red blood cells with ACK lysis buffer. These suspensions were cultured for one hour to remove non-adherent cells. When cells reached confluence, in 5-7 days, PMCs were harvested by differential trypsinization, and used for experiments or passaged further in culture. In all experiments, PMCs were used within 2 passages. Murine embryonic fibroblasts (MEFs) were harvested from day E 13.5 embryos as described elsewhere [213] and were transduced following between 2 and 5 *ex vivo* passages. Raji cells were obtained from ATCC. Primary human cells (PBMCs, bone marrow, fibrocytes, and nurse-like cells) were obtained under IRB approved protocols. Fibrocytes and NLCs were cultured as previously described.[31, 220] All cell types were cultured in DMEM, supplemented with 10% FBS and 1% penicillin/streptomycin. B16-F10 melanoma and A293T cells were purchased (American Type Culture Collection).

RT-PCR and Western Blots

PMC or MEF RNA was isolated using the RNeasy Plus kit (Qiagen) and cDNA was reverse transcribed at 50℃ using Superscript III (Invitrogen) and oligo-dT. Remaining mRNA was degraded by RNAse H (Promega). Western blots were performed on whole cell lysates separated by 4-12% SDS-PAGE (Invitrogen), blocked with 3% bovine serum albumin, and probed with the Erdr1 specific rat antibody 8A12 (Ascenion). HRP-conjugated goat anti-rat IgG (18-4818-82, Ebioscience) or donkey anti-rabbit IgG (18-8816-31, Ebioscience) were used as secondary antibodies, and blots were developed using an ECL kit (Amersham). RACE PCR was performed using the RLM-RACE kit from Amersham, according to the manufacturer's instructions, using the gene specific ("gs") primers listed in Table 3.1.

Cloning and Sequencing

Erdr1 was amplified from PMC and MEF cDNA using Accuprime GC Rich Polymerase (Invitrogen), with forward and reverse primers and thermocyler conditions described elsewhere[175], (Chapter 2). PCR products were cloned into pCR-BluntII-TOPO (Invitrogen) and transformed into TOP10 cells (Invitrogen). Kanamycin resistant clones were screened for inserts by EcoRI digestion (New England Biolabs) and sequenced using the primers provided by the manufacturer by the UNC Genome Analysis Facility.

V. Figures and Tables

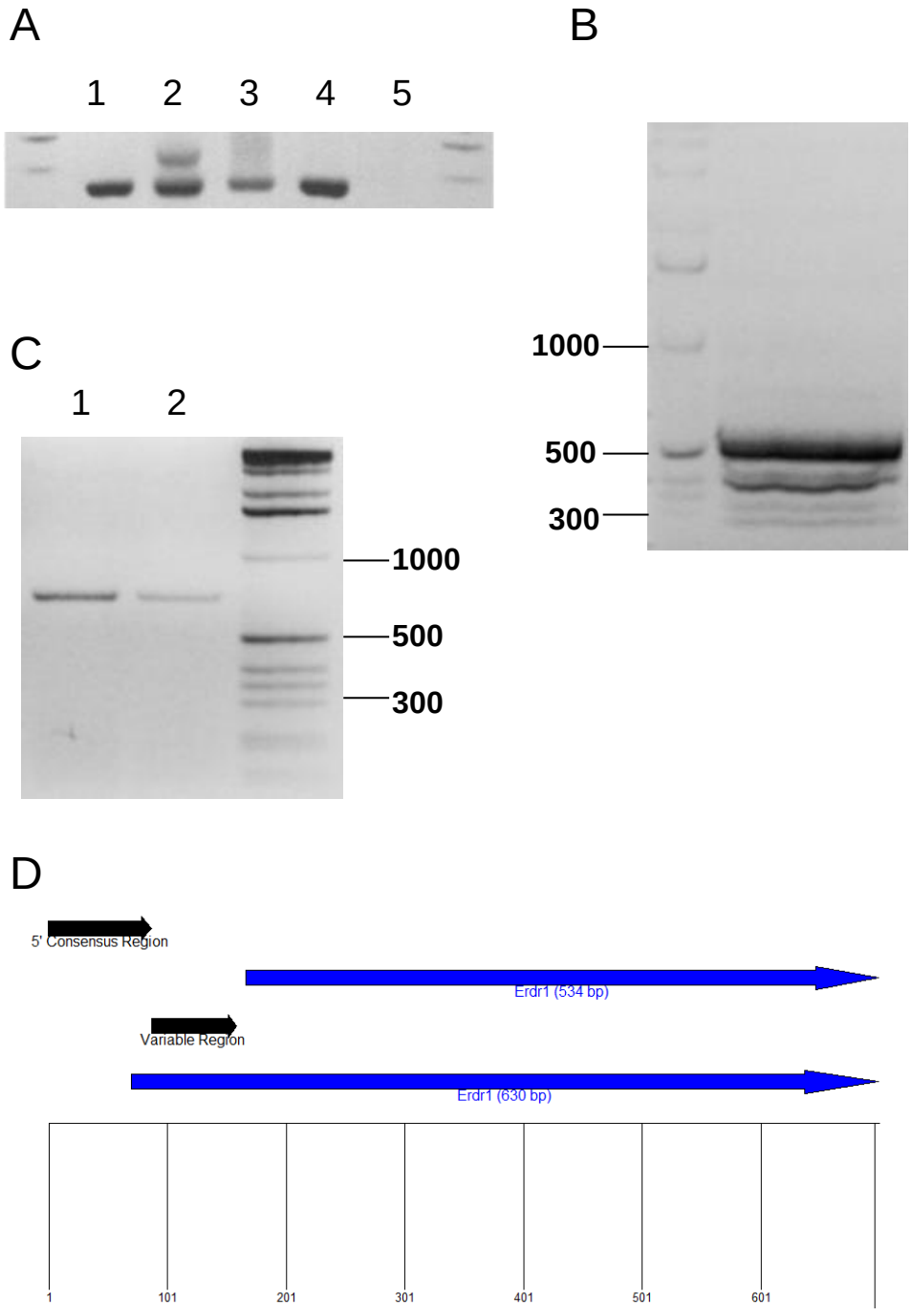


Figure 3.1 RT-PCR for Erdr1 in mouse cells.

A. Erdr1 by RT-PCR using primers for a 150 bp segment. 1. MEF 2. PMC 3. WEHI-3B 4. B16-F10 5. No template control. **B.** RT-PCR for full length *Erdr1* based on AJ539223,

using conventional protocol. Expected product size: 700 b.p. **C.** RT-PCR for full length *Erdr1* based on AJ539223, using optimized protocol. Expected product size: 700 b.p. 1. WT PMC 2. $CCR5^{-/-}$ PMC The sizes of the DNA molecular marker bands are indicated in base pairs. **D.** Schematic representation of AJ539223, with the two described ORFs, the 5' consensus region, and the variable region indicated.

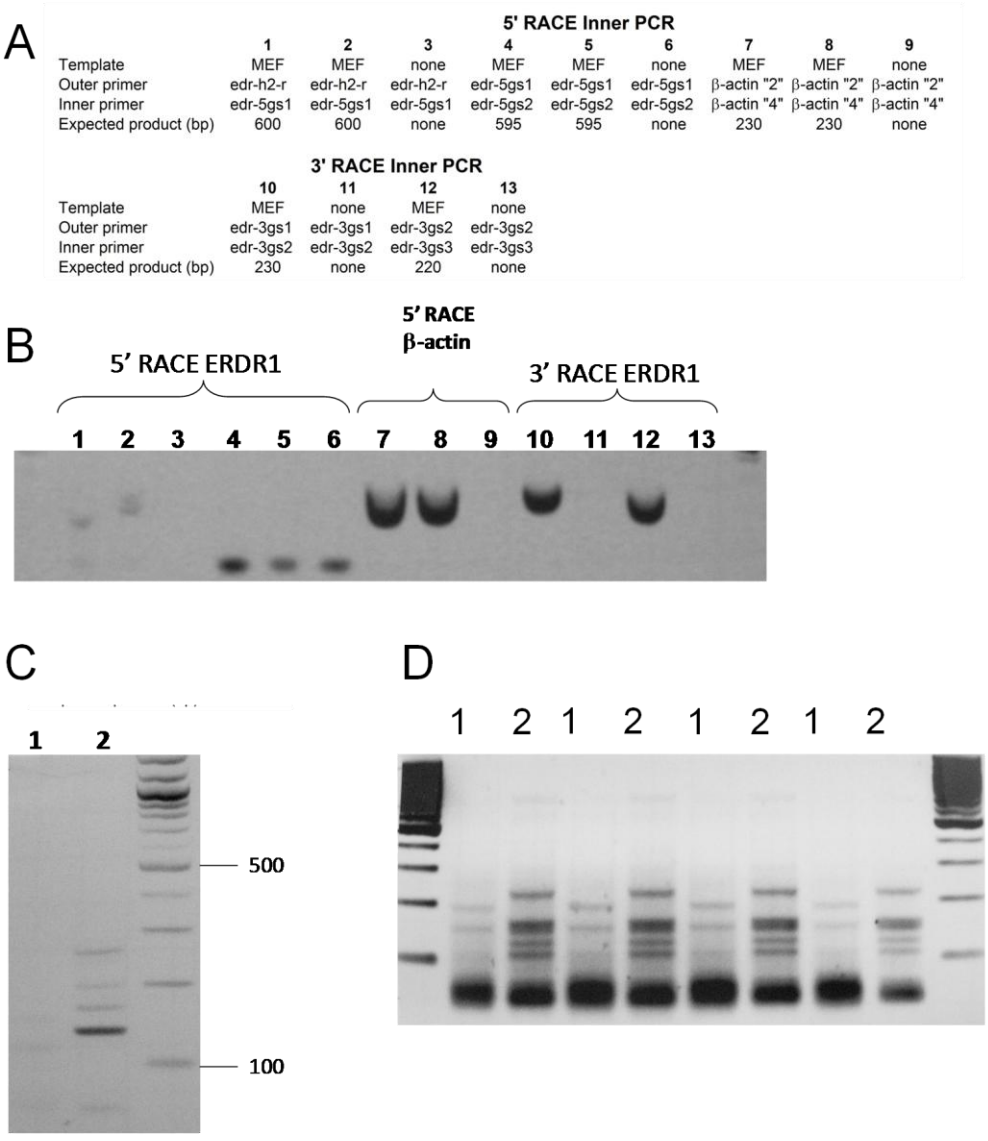


Figure 3.2 RACE PCR for *Erdr1* shows 3' but not 5' RNA integrity.

A. Primer pairs and expected sizes for RACE nested PCR. Numbers correspond to lanes on the gels below. **B.** Products of the first round of nested PCR. **C.** 5' RACE products of the second round of nested PCR. **D.** Products of the second round of 5' RACE repeated, with 4 replicates.

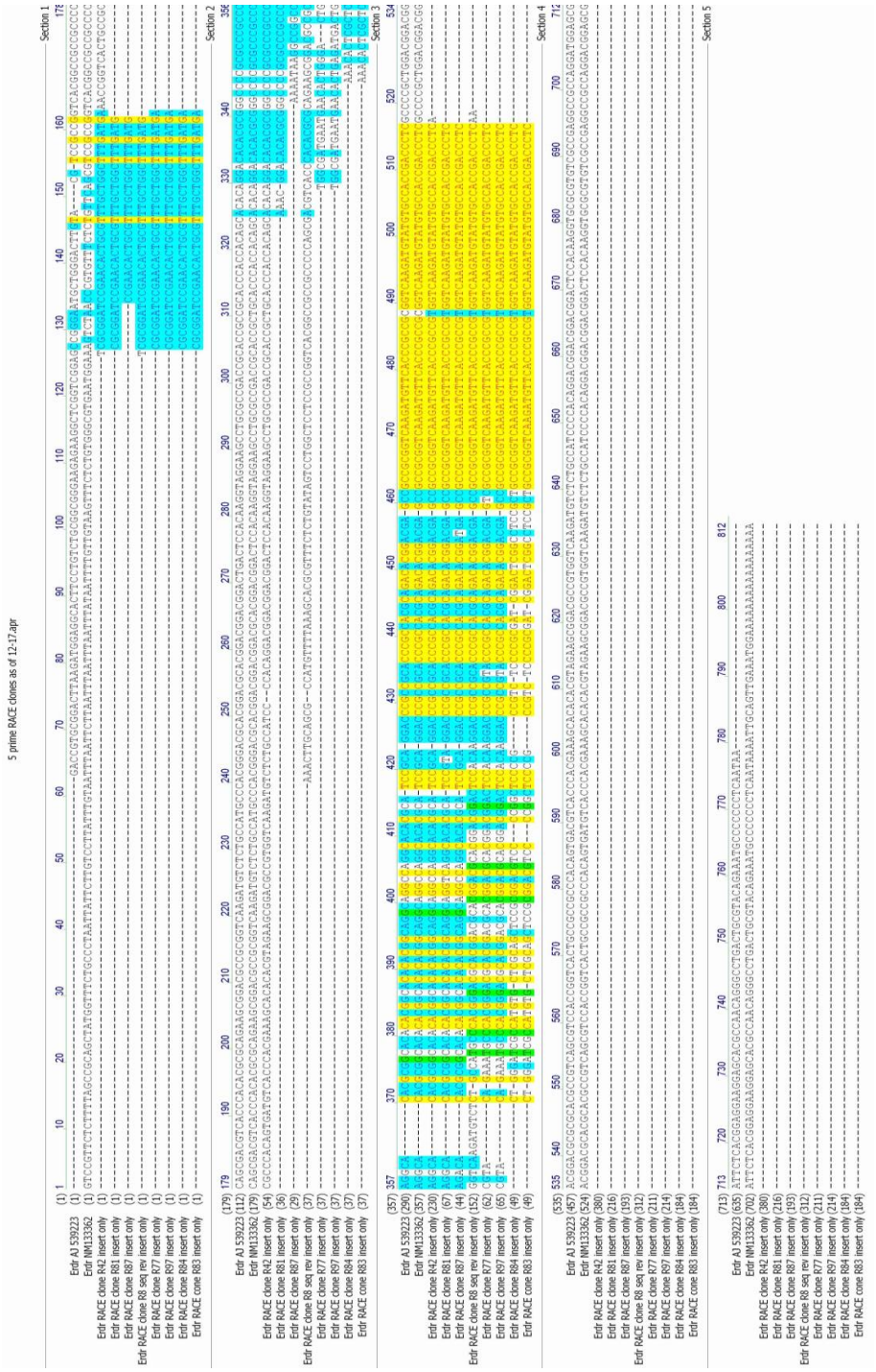


Figure 3.3 5' RACE sequences aligned with the Dormer *et al.* sequence (AJ539223) and the reference sequence (NM_133362)

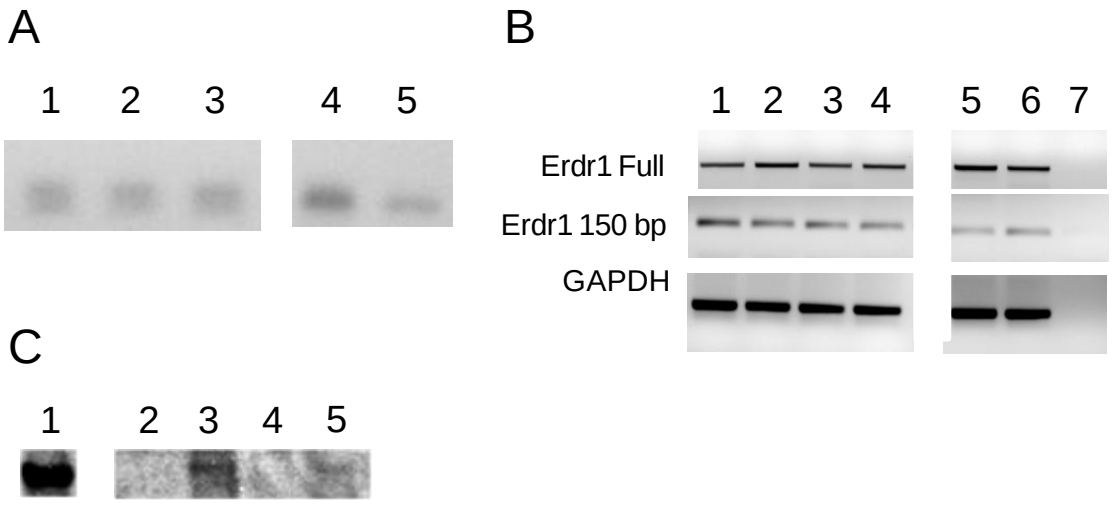


Figure 3.4 Erdr1 Expression in Human Cells.

A. Erdr1 by RT-PCR using primers for a 150 bp segment of the gene. 1-3. Human fibrocytes. 4. MEF 5. PMC. **B.** Erdr1 expression for human PBMCs by RT-PCR. 1,3,4. CLL patient. 2, 5, 6. Healthy donor. 7. No template negative control. **C.** Western blot for Erdr1 protein. 1. MEF 2. Human PBMC 3. Human PBMC 4. Human PBMC from CLL patient. 5. Human bone marrow.

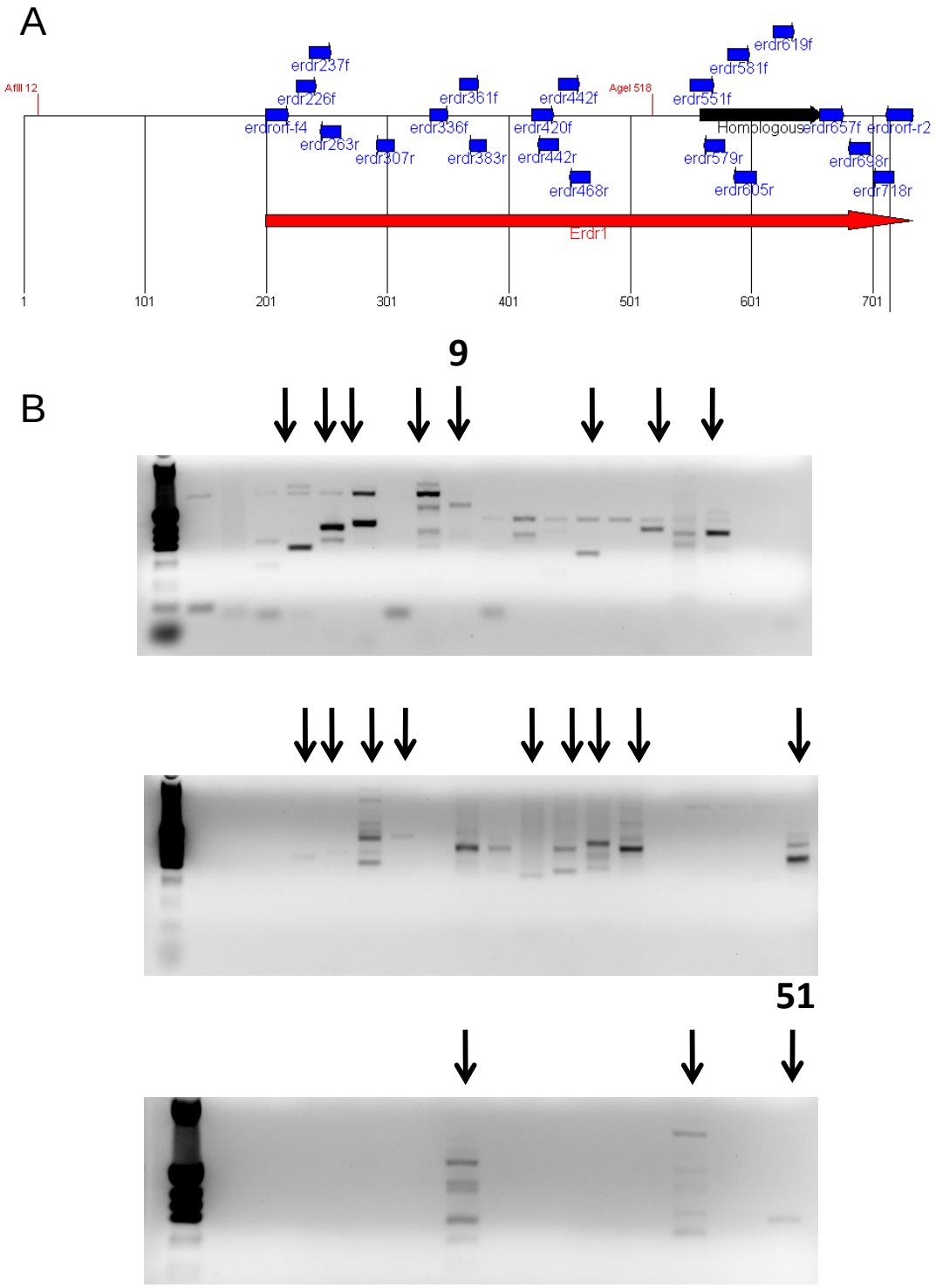


Figure 3.5 Combinatorial primer approach to identifying human Erdr1 from Raji cells. **A.** Schematic representation of primers used. **B.** RT-PCR products. Arrow: contains products near the expected size. Numbers correspond to the clones in Fig. 6.

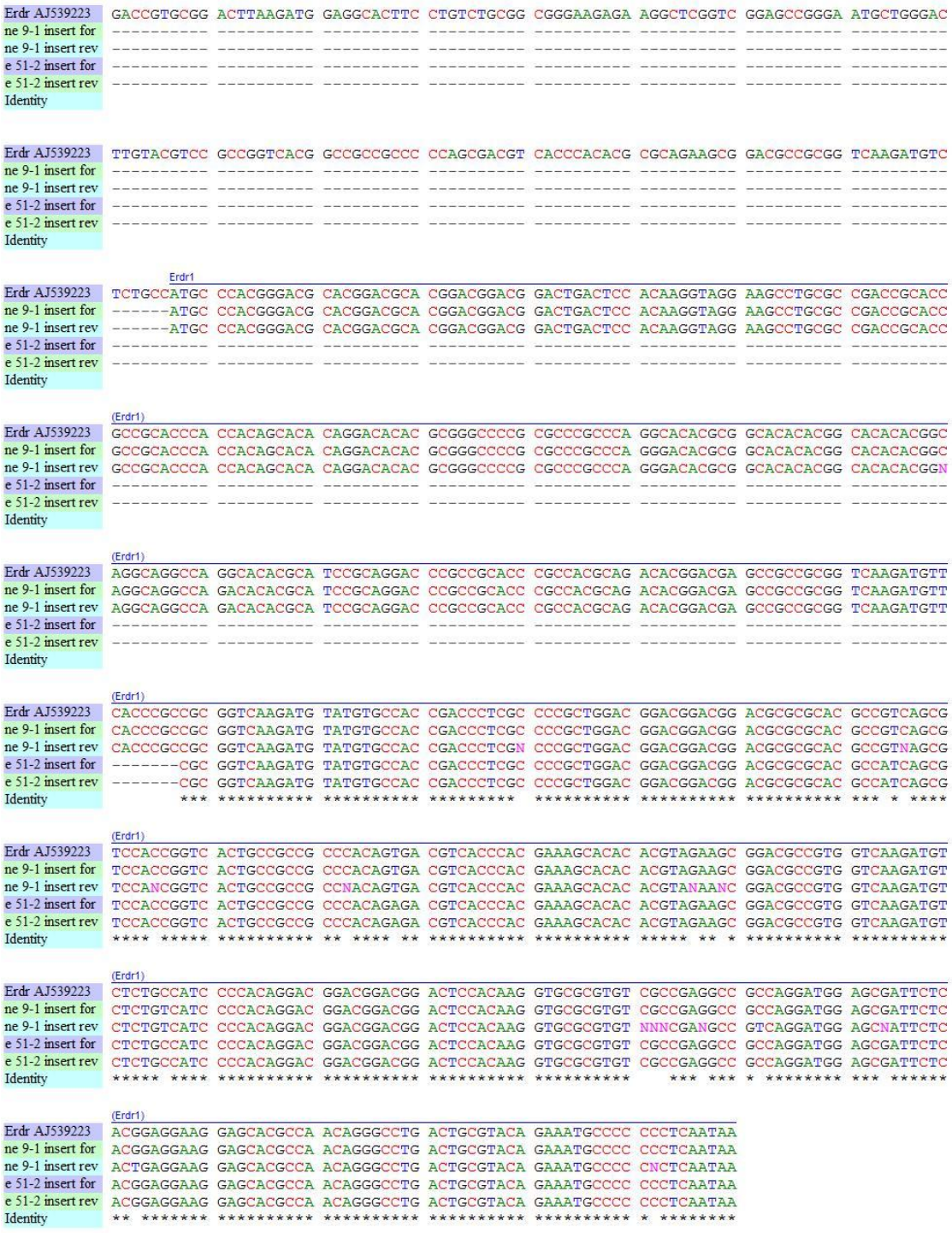


Figure **3.6** ***Erdr1*** **sequences isolated from Raji cells.**

cDNA		NLC	NLC	NLC
F Primer		229	237	361
R Primer		579	762	698
Predicted Product		350 bp	525 bp	337 bp
Clones		h202 h206 h209	h222 h226 h232	

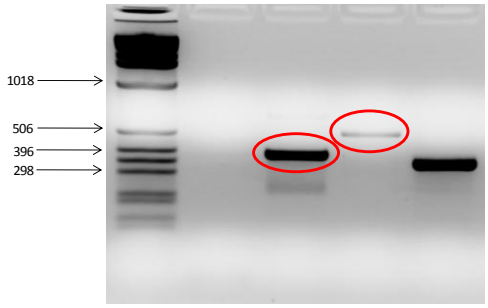


Figure 3.7 RT-PCR for Erdr1 in NLC.

Bands show PCR products using the 3 different primer sets indicated in the table. Circled bands were excised, cloned into sequencing plasmids, and sequenced. Clone numbers refer to the sequences in Fig. 3.8.

Figure 3.8 *Erdr1* sequences isolated from NLC.

Primer	Strand	Accession	5' Start position	Primer Sequence
Erdr1f	coding	AJ539223	1	GACCGTGCGGACTTAAGATGG
erdrorf-f4	coding	NM_133362	229	ATGCCCACGGGACGCACGGA
erdr226f	coding	NM_133362	226	GGACGGACGGACTGACTC
edr-3gs1	coding	NM_133362	476	CTGACTCCACAAGGTAGGA
edr-3gs2	coding	NM_133362	566	CAAGATGTATGTGCCACCGACCCT
edr-3gs3	coding	NM_133362	577	ACAGTGATGTCACCCACGAAAGCA
erdr237f	coding	NM_133362	237	ACCCCACGAAAGCACACACGTAGAA
erdr336f	coding	NM_133362	336	CACACACGGCACACAC
erdr361f	coding	NM_133362	361	GCCAGGCACACGCATC
erdr420f	coding	NM_133362	420	CGCGGTCAAGATGTTCACC
erdr442f	coding	NM_133362	442	CGCGGTCAAGATGTATGT
erdr551f	coding	NM_133362	551	CCACGAAAGCACACACGTAG
erdr581f	coding	NM_133362	581	CGTGGTCAAGATGTCTCTG
erdr619f	coding	NM_133362	619	GGACGGACTCCACAAGGT
erdr657f	coding	NM_133362	657	CAGGATGGAGCGATTCTCAC
erdr263r	non-coding	NM_133362	263	CGCAGGCTTCCTACCTTG
edr-h2-r	non-coding	NM_133362	617	CGCGTGTGTCCTGTGT
edr-5gs1	non-coding	NM_133362	596	TTGACCACGGCGTCCGCTTCTA
edr-5gs2	non-coding	NM_133362	589	ACGTGTGTGCTTTCGTGGGT
erdr307r	non-coding	NM_133362	307	TGCTTTCGTGGGTGACATCA
erdr383r	non-coding	NM_133362	383	TCCTGCGGATGCGTGT
erdr468r	non-coding	NM_133362	468	GTCGGTGGCACATACATC
erdr579r	non-coding	NM_133362	579	CGTCCGCTTCTACGTGTG
erdr605r	non-coding	NM_133362	605	GGATGGCAGAGACATCTTGA
erdr698r	non-coding	NM_133362	698	CTGTTGGCGTGCTCCTTC
erdr718r	non-coding	NM_133362	718	TTTCTGTACGCAGTCAGG
erdrorf-r2	non-coding	NM_133362	762	TTATTGAGGGGGGGCATTTCTGTA

Table 3.1 Primers used for RT-PCR.

Chapter 4
Discussion and Future Directions

I. Summary and Model

The construction and maintenance of a supportive tumor stroma is a dynamic process involving many factors and many different types of cells. In the context of metastasis, a new tumor stroma forms quickly, requiring the influx of multiple cell types, remodeling of the ECM, and cell activation and proliferation. This process can be thought of as analogous to wound formation. The dependence of metastasis on host expression of IL-6[85], S100A8 and S100A9[221], TLR2[168], CCR5[159, 170, 171] and CCL3[159] highlight the role that an inflammatory milieu plays in this process. Fibrocytes and fibroblasts respond to CCR5 ligands by migration into the metastatic lung, where they contribute to the new supportive tumor stroma.[159, 171] The data presented here implicate Erdr1 in this process.

We provide the first data that Erdr1 expression by stromal cells has an effect *in vivo*. The association of Erdr1 with CCR5 and the reduction of stromal promotion of metastasis after Erdr1 knockdown also provide a mechanism for the reduced metastasis formation in $CCR5^{-/-}$ mice. Given the anti-apoptotic activity seen in MEFs and PMCs, we propose that these cells promote metastasis by secreting Erdr1, which acts as a cell survival factor supporting the formation of a supportive stroma for metastatic nodules.

A role for stromal cell survival factors in promotion of tumor growth or metastasis has not been widely reported. Most factors influencing stromal cells specifically induce cytokine secretion or differentiation to myofibroblasts. These contribute to the formation of wound-like granulation tissue, which is often found surrounding tumors, as previously discussed. However, normal wound granulation tissue eventually clears, and this has been known for many years to depend on myofibroblast apoptosis.[222] Tumor stroma, on the other hand, has been referred to as a "wound that does not heal"[10] precisely because normal clearing does not occur. The promotion of metastasis by stromal Erdr1, then, may involve the prevention of myofibroblast apoptosis.

Of course, our evidence suggests a role for Erdr1 early in the tissue colonization process, which cannot explain long term prevention of apoptosis. PMCs injected intravenously do not persist in the lung after 5 days.[171] A recent report suggests that FGF-2, which is present in the tumor stroma and promotes fibroblast growth, can instead induce fibroblast apoptosis when cells are "pre-treated" with TGF-β1.[223] If the timing of this effect is truly important (that is, if TGF-β1 after or concurrent with FGF-2 does not have this effect), perhaps an anti-apoptotic signal from Erdr1 early in the process prevents this switch from occurring.

Another possibility is that the anti-apoptotic effect of Erdr1 persists for a long period of time. The number of apoptotic cells in our knockdown cultures did not increase appreciably until passage 2 (days 5-7) after transduction. If turnover of Erdr1 protein is low, local release in lung tissue early in metastasis may prevent myofibroblast apoptosis long enough for tumor nodules to become established. A third possibility is that overcoming the

immediate stress on stromal cells of early colonization events requires an anti-apoptotic signal.

The data also show that Erdr1 expression is linked to CCR5 signaling, particularly in fibrocytes. This presents a model in which fibrocytes, migrating into the inflammatory milieu of the new tumor site upregulate Erdr1 as they settle in the tumor stroma and become myofibroblasts. Erdr1 expression promotes their survival as well as that of the cells around them. These may not necessarily only be mesenchymal cells. The recent report by Denault *et al*. linking Erdr1 to HSC function raises the possibility that HSCs might partially depend on it to persist and promote metastasis, as has been described by Kaplan *et al*.[211] Furthermore, Kaplan *et al*. demonstrated that HSCs act to promote metastasis very early in the process, perhaps before the tumor cells leave the primary site. Therefore an early role for Erdr1 would be consistent with a mechanism mediated by HSCs.

We also show that 100-fold overexpression of Erdr1 leads to rapid cell death, which is consistent with the narrow range of anti-apoptotic activity reported by Dormer *et al*.[176] When Deneault *et al*. overexpressed Erdr1 in HSCs, it did not lead to cell death as it did for us in PMCs and MEFs. Perhaps the detriment seen to cell survival at high doses varies by cell type. The promoter in their expression vector was PGK, and perhaps this led to more physiological expression levels than the promoters we used. Another difference between the expression vectors is the *Erdr1* sequence used. The sequence in our vector begins with the 5' consensus sequence of mRNA species, as shown in Chapter 2. With the extra 34 bp that we consistently found, this means that our expression vector included 200 bp of untranslated sequence 5' to the start site. The sequence in the vector used by Deneault *et al*. only includes the 534 bp transcript. Further studies would be needed to determine if overexpression of a

transcript with the 200 bp 5' untranslated region (UTR) specifically influences cell viability. However, we favor the interpretation that the vectors differed in dose effect. Deneault *et al*. did not quantify Erdr1 expression in the transduced HSCs, precluding a direct comparison of doses achieved.

The negative correlation of Erdr1 expression with differentiation found in multiple microarray studies does not necessarily imply that Erdr1 inhibits differentiation, but it does suggest the possibility that Erdr1 inhibits the differentiation of fibrocytes into fibroblasts in our metastasis model. We believe this is not the case. We did not find higher Erdr1 expression in fibrocytes than in other PMC populations *in vitro*, except when stimulated by CCL4 (see Chapter 2). Furthermore, knockdown of Erdr1 did not result in fewer fibrocytes. In fact, in some knockdown PMC or MEF cultures, the relative size of the fibrocyte population grew over time, which appeared to be a consequence of more apoptosis in the other populations (unpublished observations). This phenomenon was not seen in every Erdr1 knockdown culture, but it does emphasize that differentiation is probably not a mechanism to explain our results, and if it were, it would seem that Erdr1 promotes, rather than inhibits, fibrocyte differentiation to fibroblasts.

Our data also extend previous findings on the expression of Erdr1, particularly expression in stromal and human cells. It appears unlikely that PMCs or MEFs express the 630 bp ORF characterized by Dormer *et al*., since every sequenced clone from these cells contained a 34 bp insert interrupting the reading frame of that transcript. Furthermore, the sequence we have obtained produces a functional protein that reduces stromal cell survival in high concentrations. We have confirmed, by cloning and sequencing *Erdr1* PCR products with multiple different primer sets, the previous report that *Erdr1* appears to be identical in

humans and mice. The identification of this sequence in NLCs extends the list of cell types that express Erdr1 to one that is phenotypically similar to the fibrocytes in our studies, and that is clinically relevant.

II. Future Directions and Clinical Relevance

Though few studies have focused on Erdr1, the evidence to date establishes it as an important subject for future study. We and others have shown that it functions as an antiapoptotic survival factor, with *in vivo* relevance to HSC activity as well as metastasis. Functional genomics data also implicate Erdr1 in progenitor cell and stromal cell activity, in addition to development, immunity, and neuronal protection/degeneration. We have shown it to be induced through CCR5, which might be mediated by CREB, and other data suggest positive regulation by E2F1 and negative regulation by PKBα (see App II). Given these clues, many important questions remain.

Regarding metastasis, what other cell types are influenced by stroma-derived Erdr1? Given the microarray data implicating Erdr1 in immune activation, it might be possible that it plays such a role at the tumor site. If so, is there a requirement for cell-intrinsic expression, or can secreted Erdr1 mediate the effect? To answer these questions, new tools are needed. Genetic models in combination with recombinant protein and a neutralizing antibody would help to determine the cell types requiring Erdr1 intrinsically or extrinsically. Given the data that Erdr1 might be important during development, the genetic models would likely have to be conditional to a specific cell type or inducible in adulthood. A CRE-loxP conditional knockout of Erdr1 in stromal cells using the FSP-1 promoter would be useful not only in this

model system, but also in fibrosis models where the CCR5-mediated induction of Erdr1 in fibrocytes might also be important.

In order to engineer a conditional knockout mouse, the genomic context of the gene must be verified. While the BAC clone with the distal X chromosome sequence does give sufficient information to flank *Erdr1* with loxP sites, this sequence has not yet been annotated on the mouse genome. Confirmation of this position using some of the BAC clone sequence as a probe for fluorescent *in situ* hybridization (FISH) would be necessary, and screening a mouse genomic library for *Erdr1* to confirm the sequence would be ideal.

Similar studies of the human genome would also be useful. As mentioned previously, the human genome databases do not contain a sequence for *Erdr1*, so genomic context for design of a FISH probe is not available. In 2004, there remained 357 gaps in the human genome, which Cole *et al*. estimated might contain about 2000 previously unidentified human genes.[224] Gaps still exist today, as is illustrated by the recent identification of 720 new human genomic loci, 156 of which potentially encode genes.[225] Factors that might inhibit the discovery of transcripts by high throughput methods include short open reading frames, single exon genes, or locations within heterochromic regions (found in the distal or central regions of chromosomes).[224]

These factors apply to *Erdr1*, suggesting why there is a scarcity of genomic sequence. Furthermore, our identification of a high fidelity cDNA transcript sequence required significant optimization of RT-PCR protocols, suggesting that high throughput methods might also miss *Erdr1* in cDNA libraries. With the method we have developed, a human genomic library could be screened for *Erdr1*, thus providing the flanking sequences

necessary for FISH probe design and for genomic annotation. Alternatively, probing a Southern blot of human genomic DNA with the ORF sequence we isolated could identify genomic *Erdr1*, provided the human sequence, like the mouse, contains no introns within the ORF.

Future studies could also identify the signaling pathways that might regulate Erdr1. Genetic or pharmacological inhibition of mediators downstream from CCR5 would further test the hypothesis that CCR5 induction of Erdr1 depends on this pathway. Protein kinase B (PKB), also known as Akt, is a downstream mediator of phosphoinositol-3 kinase (PI3K) signaling, and has been shown to mediate the induction of antiapoptotic genes upon stimulation of macrophages through CCR5.[226] However, PKBα knockout MEFs have higher Erdr1 expression than WT MEFs (App. II, 39).[227] This does not rule out Erdr1 CCR5-induced upregulation through PKBβ or PKBγ, but PKBα appears an unlikely candidate.

Isolation and cloning of the Erdr1 promoter into a reporter construct, with sequential mutation of putative transcription factor binding sites, would help to elucidate the regulation of Erdr1 in multiple contexts. Based on the genomic sequence from the BAC clone RP24-143B12, there appears to be a CREB binding site within a predicted promoter region at position -1050 bp relative to the start site (PROSCAN weight = 2.55, promoter score = 76.09, cutoff = 53.00; i.e., promoters with scores above 53 have a false positive rate of 1 in 14,000 bp).[228] There are other potential transcription factor binding sites in this putative promoter region, including EGR-1 (weight = 5.74), Sp1 (3.36), and GCF (2.28).[228] Of these, only CREB has been previously linked to CCR5.[229] Furthermore one microarray experiment

implicates CREB binding protein (CBP) and its associated CREB co-factor p300 in Erdr1 regulation.[230] (App. II, 43, 45)

Clues on the regulation and biochemical mechanism of action of Erdr1 could also be revealed by identification of Erdr1 binding partners. Since the sequence contains no conserved domains, this would need to be an unbiased approach. Development of a neutralizing antibody would allow co-immunoprecipitation (co-IP) of Erdr1 with binding partners, and could then be denatured, separated by two-dimensional gel electrophoresis and identified by mass spectrometry. Alternatively, a proteomics array could be implemented, whereby plate-bound Erdr1 is allowed to bind proteins from a cell lysate, which are then probed by an array of protein-specific antibodies. A systems biology approach could also be employed to identify genes or proteins differentially regulated in Erdr1 knockdown cells with or without stimulation by recombinant Erdr1, providing information on the general downstream effects of Erdr1 signaling.

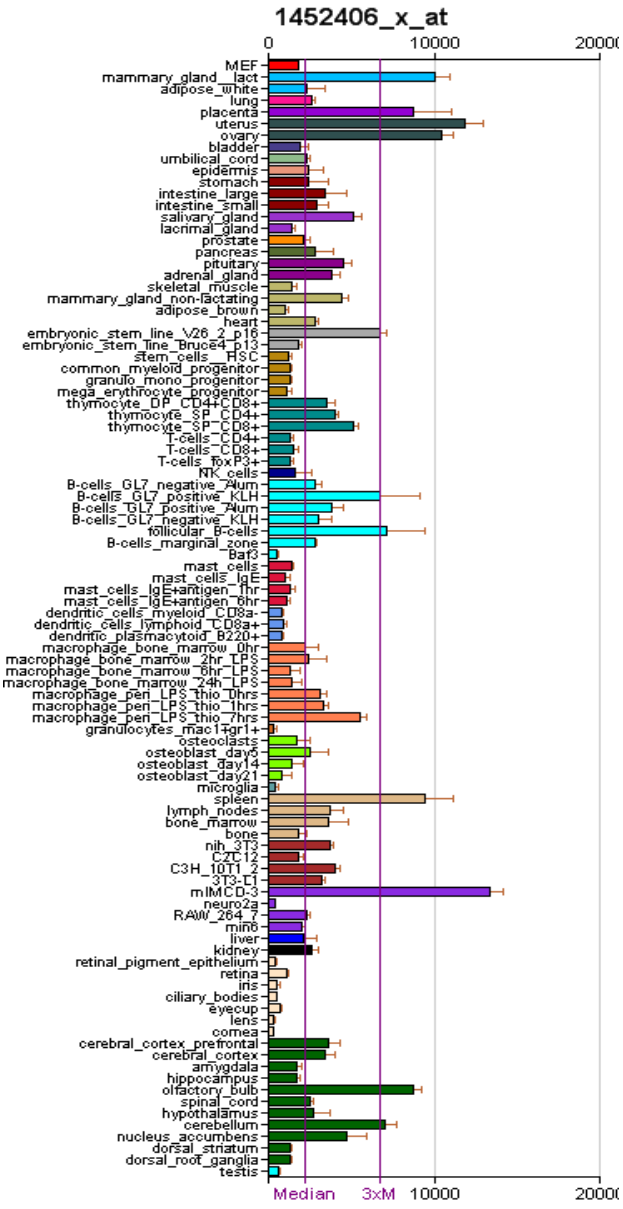
Finally, the development of small molecule inhibitors of Erdr1 would allow evaluation of the efficacy of Erdr1 blockade as a therapeutic modality. The strategy of targeting stroma-derived factors to complement cancer therapy has begun to show some benefit in the clinic, particularly with the targeting of VEGF-mediated angiogenesis (for review, see [136]). However, this strategy has not been as efficacious as hoped, partially due to compensation with other pro-angiogenic factors, such as FGF[231] and PDGF-C[141]. It is possible that blocking a stromal cell survival factor, such as Erdr1, might reduce such compensation by stromal cells, and increase the efficacy of stromal targeting.

As mentioned previously, a small molecule inhibitor for CCR5 is already clinically available for use in HIV patients.[173] The findings presented here strengthen previous data implicating CCR5 in metastasis by providing another factor downstream from CCR5 that assists the stromal promotion of metastasis. According to our model, the influx of fibrocytes into a new metastatic site via CCR5, and the subsequent ECM modification by MMP-9 and stromal cell support by Erdr1 are important for successful metastatic colonization. Blocking CCR5, then, could interfere with metastasis early in the process, possibly increasing disease-free survival for patients. Moreover, this strategy targets non-neoplastic cells that have not yet been influenced by the tumor microenvironment, reducing the possibility of the development of drug resistance.

In summary, stromal cells play an important role in the promotion of tumor growth and metastasis. We have identified the involvement of a recently characterized gene, Erdr1, in the promotion of metastasis by stromal cells. Our data shows that stimulation of CCR5 on fibrocytes induces Erdr1, which acts as an antiapoptotic survival factor to support the pro-metastatic stroma. We have also shown that this involves the expression of a 534 bp Erdr1 ORF, which is conserved at the cDNA level in humans and mice. Our data are consistent with other studies showing that Erdr1acts as a survival factor, and provide the first evidence that it promotes disease *in vivo*. Given this and other potential roles, Erdr1 requires further investigation for the translation of these findings into clinical benefit.

Appendix I

BioGPS Expression Profile of Erdr1[177, 232]



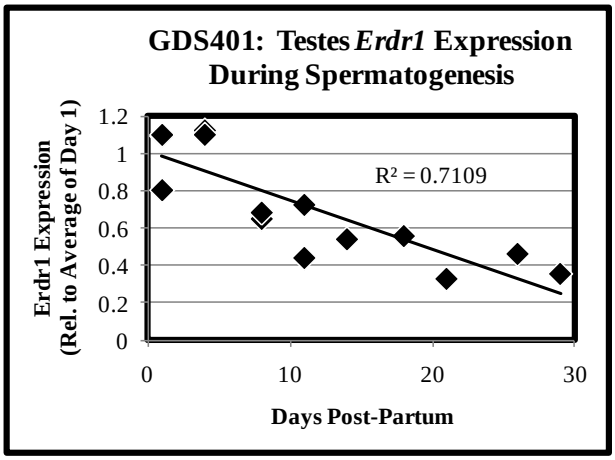
Appendix II
Summary of cDNA Microarray Data Involving Erdr1

The Gene Expression Omnibus (GEO) database was searched using the term "Erdr1." Results were included in this table if the change in expression was greater than 2-fold up or down. Results are loosely arranged in topic areas. Within each table, results are ordered from greatest fold upregulation to greatest fold downregulation, except that results from the same study are grouped together. These data provide general insights into otherwise unknown associations between Erdr1 and various phenomena. However, most of these findings were not followed up by other methods, and so they are presented only as a general context for direct experimental data.

See: http://www.ncbi.nlm.nih.gov/geo/

Arrays Associating *Erdr1* with Less-Differentiated Cells						
#	**GEO Data Set**	**Cells /Tissue**	**Comparison (Denominator)**	**Experimental Group (Numerator)**	***Erdr1* Fold Change**	**Ref**
1	GDS1560	Skin	Wild type	Induction of conditional β-catenin transgenic (local de-differentiation)	12.89	180
2	GDS2398	Hematopoietic cells	Granulocytes	Hematopoietic stem cells	9.75	178
3	GDS2398	Hematopoietic cells	Granulocytes	Short term repopulating hematopoietic progenitors	9.49	178
4	GDS2398	Hematopoietic cells	Granulocytes	Long term repopulating hematopoietic progenitors	4.85	178
5	GDS2718	Cultured stem cells	Hematopoietic	Embryonic	4.19	247
6	GDS1010	Bone marrow	Main poulation	Side Population (enriched for hematopoietic stem cells)	3.41	179
7	GDS2103	Stomach	WT + H. felis	Gastrin transgenic (pro-tumorigenic) + H. felis	2.07	248
8	GDS586	C2C12 cells	Proliferating C2C12	Fully differentiated myotubes (day 10)	0.47	181
9	GDS2412	C2C12 cells	Regular C2C12 culture conditions	Induction of myotube differentiation	0.45	182

Arrays Implicating *Erdr1* in Development						
#	**GEO Data Set**	**Cells /Tissue**	**Comparison (Denominator)**	**Experimental Group (Numerator)**	***Erdr1* Fold Change**	**Ref**
10	GDS2577	Liver	Adult liver after partial hepatectomy (10 timepoints combined)	Fetal liver (Day 10.5 to 16.5 combined)	6.40	195
11	GDS812	Embryos	2 cell stage	8 cell stage	4.60	194
12	GDS812	Embryos	Blastocyst	8 cell stage	3.16	194
13	N/A	Embryos day 10.5	Male	Female	2.24	193
14	N/A	Embryos day 11.5	Male	Female	2.09	193
15	GDS401	Testis	1 day post partum	29 days post partum	0.36 (see chart)	233
16	GDS2704	Smooth muscle cells	Wild type	CHF1/Hey2 knockout (defective vascular development and injury response)	0.27	234



Arrays Associating *Erdr1* with Stromal Cells						
#	**GEO Data Set**	**Cells /Tissue**	**Comparison (Denominator)**	**Experimental Group (Numerator)**	***Erdr1* Fold Change**	**Ref**
17	GDS2818	Intraabdominal adipose tissue	Adipocyte fraction	Stromovascular fraction	5.30	184
18	GDS2818	Subcutaneous adipose tissue	Adipocyte fraction	Stromovascular fraction	4.69	184
19	GDS1492	Lung	CH3/HeJ female	C57/BL6 female (more susceptible to fibrosis)	2.38	235
20	GDS1492	Lung	A/J female	C57/BL6 female (more susceptible to fibrosis)	2.20	235
21	GDS1492	Lung	CH3/HeJ male	C57/BL6 male (more susceptible to fibrosis)	1.92	235
22	GDS1492	Lung	A/J male	C57/BL6 male (more susceptible to fibrosis)	1.82	235
23	GDS2421	MEFs	Media	FGF-2 40 ng/mL	2.09	
24	TBD	Lung	WT 6 hr post tumor injection	CCR5-/- (fewer metastases) 6hr	0.27	See Ch. 2
25	TBD	Lung	WT 24 hr post tumor injection	CCR5-/- (fewer metastases) 24 hr	0.37	See Ch. 2
26	TBD	Lung	WT 48 hr post tumor injection	CCR5-/- (fewer metastases) 48 hr	0.28	See Ch. 2

Arrays Involving *Erdr1* and Inflammation or Immunity						
#	**GEO Data Set**	**Cells /Tissue**	**Comparison (Denominator)**	**Experimental Group (Numerator)**	***Erdr1* Fold Change**	**Ref**
27	GDS728	CD8+ T cells	Naive CD8+ CD44low	Memory CD8+ (tetramer+ CD8+ 80 days post LCMV infection)	34.79	190
28	GDS1254	Embryonic stem cells	Wild type	SOCS3 knockout	7.54	236
29	N/A	Lung	0 hr post LPS exposure by nebulizer	6 hr post LPS exposure by nebulizer	2.70	237
30	N/A	Somatosensorial Cortex	WT Day 4 post brain cryolesion	IL-6 knockout day 4 post brain cryolesion	2.4 - 3.6 *	187
31	N/A	Somatosensorial Cortex	WT Day 16 post brain cryolesion	IL-6 knockout day 16 post brain cryolesion	1.8 - 3.0 *	187
32	GDS325	BM-derived macrophages	Wild type	SOCS3 knockout	2.03	192
33	E-MEXP-284 (ArrayExpress)	Hippocampus	170 days post mock infection	170 days post ME7 Infection (Prion model, period of peak neuronal loss)	2.00	108
34	GDS352	CD4-CD8- splenic dendritic cells	Ex vivo	Cultured in vitro	1.97	238
35	GDS787	Cerebral cortex	Wild Type	Presenilin knockout (Alzheimer's model)	0.49	239
36	GDS2903	Tumor	TC-1 cells not passaged in vivo (immune susceptible)	TC-1 cells selected for immune resistence by 3 in vivo passages	0.16	240
37	GDS432	BM-derived dendritic cells	Wild type	CIITA knockout	0.11	241
			* Numerical values not reported; entries estimated from heat map			

Other Arrays Suggesting Regulation of *Erdr1*						
#	**GEO Data Set**	**Cells /Tissue**	**Comparison (Denominator)**	**Experimental Group (Numerator)**	***Erdr1* Fold Change**	**Ref**
38	GDS575	Prostate epithelial cells	Wild type	Rb knockout	3.84	245
39	GDS1784	MEFs	Wild type	PKBa knockout	3.42	233
40	GDS46	NIH 3T3 embryonic fibroblasts	β-Gal expressing adenovirus	E2F1 expressing adenovirus	2.78	74
41	GDS2406	Lung day E 18.5	Wild type	Nmyc transgenic mouse	2.29	246
42	GDS2757	Fetal liver erythroblast islands	Wild type	Rb knockout	2.23	247
43	GDS2160	Fibroblasts	Wild type	Mutation of CH1 domains in CBP and p300	0.53	229
44	GDS2820	Skin	Wild type	Cannabinoid receptors CB1 and CB2 double knockout	0.47	249
45	GDS2160	Fibroblasts	Wild type + Hypoxia	Mutation of CH1 domains in CBP and p300 + Hypoxia	0.43	229
46	GDS703	Extraocular muscle	Wild type	Mdx mice (model for Duchenne muscular dystrophy)	0.36	245
47	GDS703	Gastrocnemius / Soleus	Wild type	Mdx mice (model for Duchenne muscular dystrophy)	0.28	245
48	GDS2630	Spleen	Wild type	Nix knockout (unrestrained erythropoiesis)	0.05	246

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