Supplemental Data

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

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Supplementary Experimental Procedures

RT-PCR

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*. The following primers were used: *Erdr1* specific primers: Forward 5'-CCGCCGCGGTCAAGATGTATGT-3' and Reverse 5'-TTGACCACGGCGTCCGCTTCT A-3'; *β-actin* specific primers: Forward 5'-TTCTTTGCAGCTCCTTCGTT-3' and Reverse 5'-GAGTCCTTCTGACCCATTC-3'; *SDHα* specific primers: Forward 5'-GGAACACTCCAAAAACAGACCT-3' and Reverse 5'-CCACCACTGGGTATTGAGTAGAA-3'.

Cloning and Sequencing

Full length *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 TTATTGAGGGGGGGCATTTCTGTA-3', and 40 cycles of 95 ^{o}C for 30 sec, 60 ^{o}C for 30 sec, and 72 ^{o}C for 2 minutes(adapted from (13) 26). PCR products were cloned into pCR-BluntII-TOPO (Invitrogen) and TOP10 cells (Invitrogen) were transformed. Kanamycin resistant clones were screened for inserts by EcoRI digestion (New England Biolabs) and were 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 EF1α promoter was obtained by a restriction cloning strategy, involving the removal of the CMV promoter from pLenti7.3-*Erdr1*, the removal of the EF1α promoter from pEF-DEST (Invitrogen), and the ligation of the EF1α promoter into the promoterless pLenti7.3-*Erdr1* plasmid.

Packaging of shRNA Vectors

Short interfering RNA sequences were obtained from Dharmacon and were validated *in vitro* (No Target shRNA: D-001810-01-05, *Erdr1* shRNA #1: J-053706-09, *Erdr1* shRNA #2: J-053706-11) 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 (23). Supernatant containing recombinant virus was harvested at various time points between 36 and 72 hours post-transfection and was 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, which was followed by re-suspension in PBS. Viral titer was determined by transfecting NIH 3T3 cells (ATCC) and assaying for GFP expression by flow cytometry 48 hours after transfection.

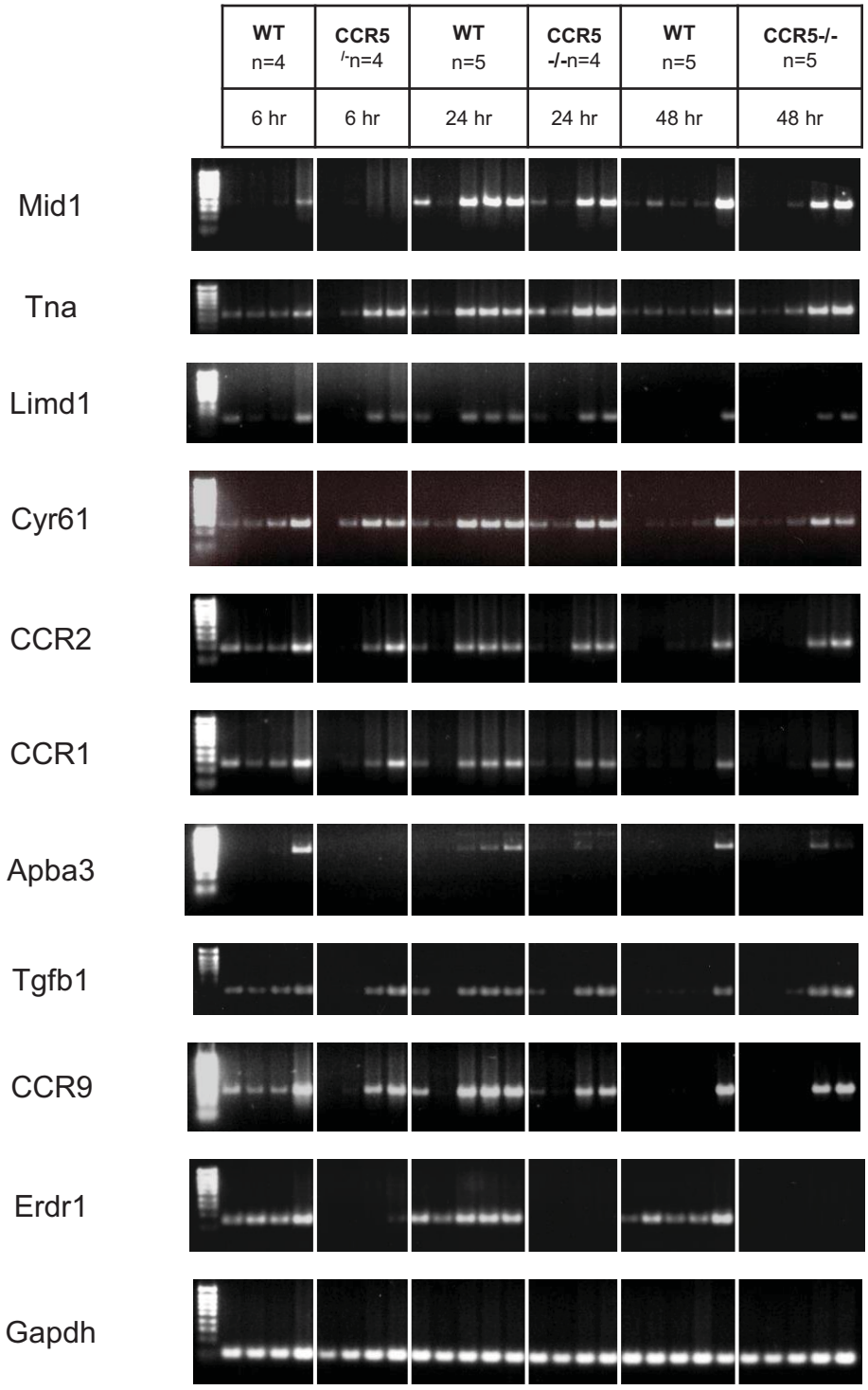

Figure S1. Semi-quantitative RT-PCR for genes differentially regulated at 6, 24, and 48 hours after injection by B16 F10 injection. Semi-quantitative PCR was applied to the unpooled samples from WT and CCR5$^{-/-}$ mice following injection with 7.5 x10^5 B16 F10 cells. This technique was applied to genes that were differentially expressed by Affymetrix analysis applied to the pooled samples. Of the 11 genes studied, only *Erdr1* showed consistent expression in WT compared with CCR5$^{-/-}$ mice.

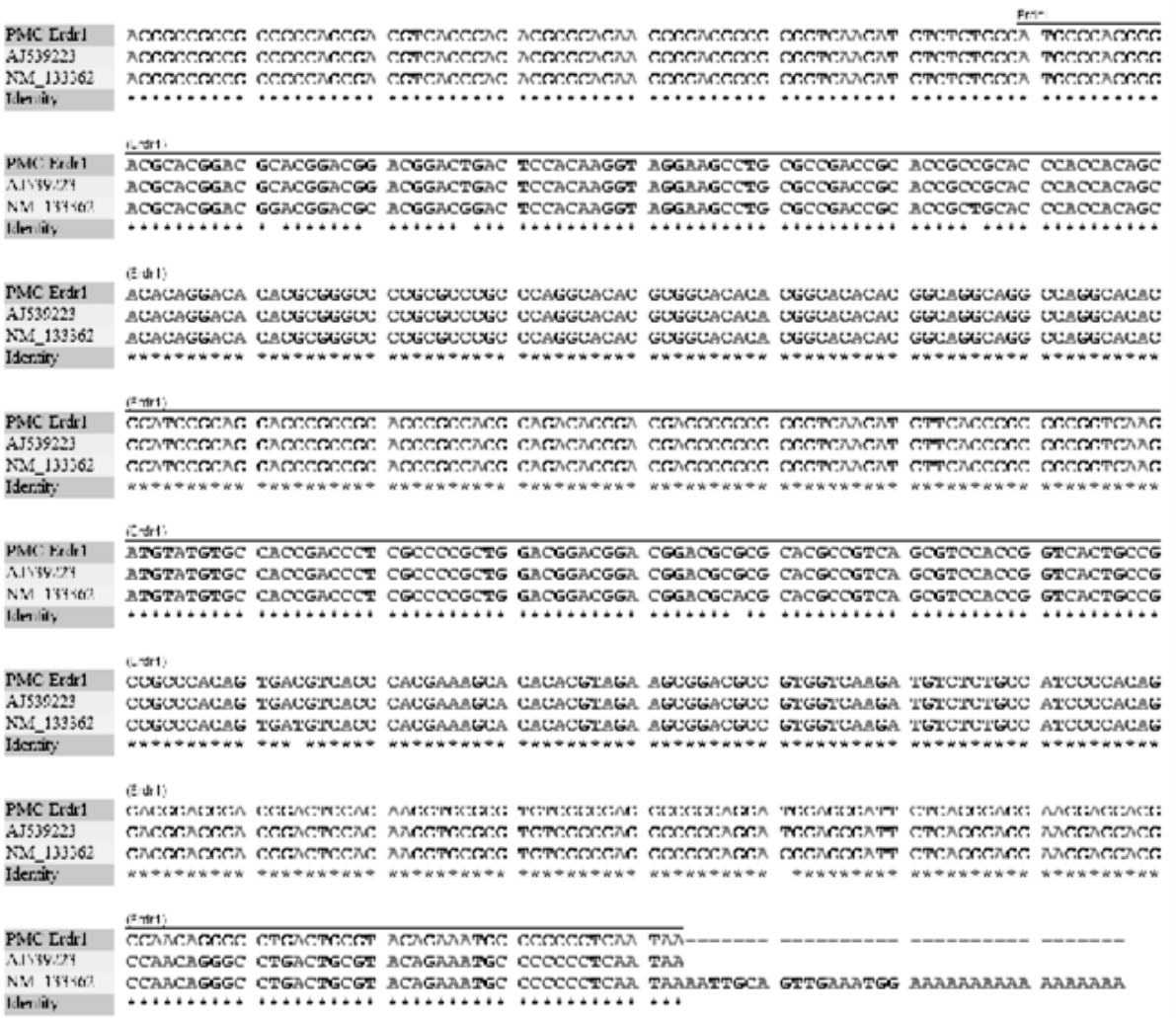

Figure S2. Sequence alignment comparing the coding sequence for *Erdr1* from cDNA extracted from PMCs with the consensus published sequences. The top line is the sequence of *Erdr1* taken from cDNA extracted from PMCs. This sequence was identical in 20 clones taken from multiple PMC cultures. The second line is the sequence as isolated from WEHI-3; the third line is the NCBI consensus sequence.

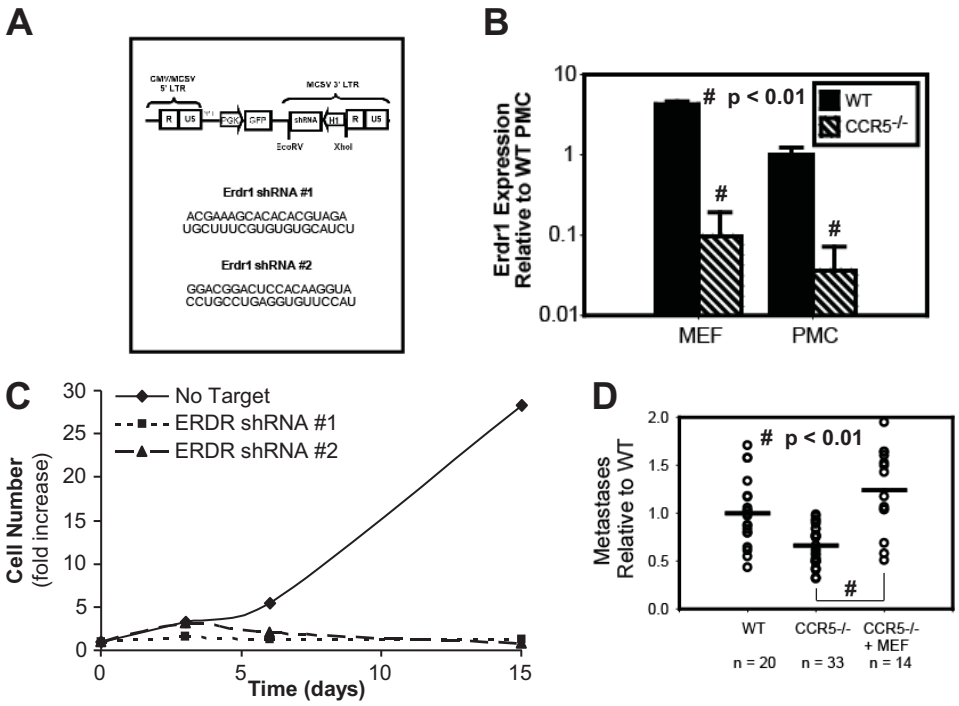

Figure S3. MEFs promote tumor metastasis. (A) Map of retroviral construct used for shRNA knockdown, and shRNA sequences. (B) *Erdr1* expression in WT and CCR5$^{-/-}$ MEFs and PMCs by real-time RT-PCR. Results were normalized to *SDHα* and were expressed as fold expression relative to WT PMCs. WT MEFs and PMCs express more *Erdr1* than their CCR5$^{-/-}$ counterparts. (C) PMCs transduced with shRNA to *Erdr1* do not expand in culture. The graph depicts cellular expansion as fold increase over a two week period. PMCs transduced with shRNA knockdown vectors showed a relative fold-increase of 1.17 ± 0.22 and 0.85 ± 1.05 respectively. Control transduced PMCs expanded by 28.3 ± 2.23 fold. (D) WT MEFs increase metastasis formation in CCR5$^{-/-}$ mice. The graph shows the number of metastatic colonies as expressed by a ratio relative to the mean number of metastases in WT mice. CCR5$^{-/-}$ mice were injected with 4x10^5 MEFs 24 hours prior to receiving B16-F10 cells.