Supplemental Materials

1. Construction of 12(S)-LOX over-expressing Caco2-LOX cells

Choice of cell lines

RNA was isolated from the colorectal cancer cell lines Caco2, SW480, and SW620. 12(S)-LOX mRNA was highest in SW620 cells that represent a metastatic phenotype and lower in Caco2 and SW480 cells obtained from primary tumours. When 10µM AA was added to the cultures SW620 cells secreted 2-3-fold more 12(S)-HETE into their medium than SW480 and Caco2 cells (figure 1s).

Production of 12(S)-LOX over-expressing clones

$2x10^6$ Caco2 cells were transfected with 16µg pcDNA3 carrying the human 12(S)-LOX cDNA by electroporation using the Nucleofector Kit T (Amaxa Biosystems, Gaithersbury, MD) and the protocol for Caco2 cells provided by the manufacturer. Controls were transfected with equal amounts of vector DNA or the control plasmid pEGFP-c1 (Clontech, Mountain View, CA) expressing green fluorescent protein (GFP). DNA uptake as judged by GFP-Fluorescence using FACS analysis was >50% in all experiments. Populations stably over expressing 12(S)-LOX were selected by cultivating cells in the presence of 0.2mg/ml G418.

Over-expression was verified by standard RT-PCR using primers specific for RNAs resulting from the endogenous gene as well as primers recognizing total 12(S)-LOX mRNA (table 1s; figure 2s a). LOX activity was determined by measuring the amount of 12(S)-HETE secreted into the supernatant within 24 hours (figure 2s b).

To assess the impact of 12(S)-LOX over-expression on growth of both SW480 and Caco2 cells were cultivated in the presence of 10µM AA and cell viability determined for 3

consecutive days by neutral red uptake (figure 3s). The fatty acid was toxic for cells, but LOX-over-expressing clone pools had a survival advantage.

2. Knock-down of 12(S)-LOX gene expression

To suppress 12(S)-LOX expression we introduced anti-sense oligonucleotides directed against 12(S)-LOX [26]. In experiments using Caco-LOX and SW480-LOX cells a second dose of anti-sense was introduced 48 hours after the first (figure 4s a). Knock-down efficiency was assessed by RealTime RT-PCR and 12(S)-HETE ELISA. For SW620 cells knock-down was efficient reducing mRNA-levels below 20% even after the first transfection (figure 4s b). SW480-LOX and Caco-LOX cells were more resistant against knock-down, but 12(S)-LOX expression could be reduced to 55-70% of controls transfected with scrambled control nucleotides 48 hours after the second transfection (figure 4s c, d). 12(S)-HETE production dropped beneath detection level in SW620 and SW480-LOX cells (data not shown) and was reduced to 50% in Caco2-LOX cultures (figure 4s e).

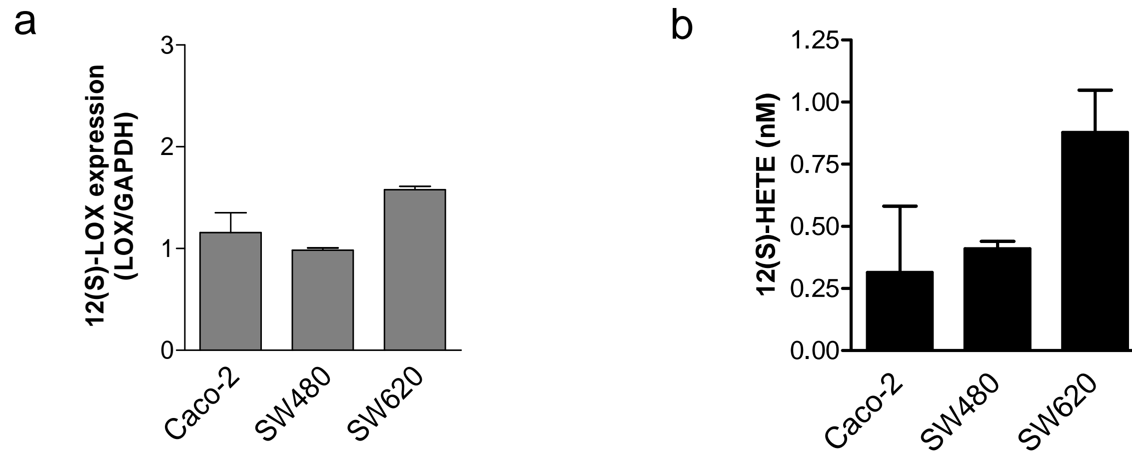

Figure 1s: Expression of 12(S)-LOX in human colorectal tumours and cell lines
a: RNA was isolated from exponentially growing Caco2, SW480, and SW620 colon carcinoma cells and 12(S)-LOX expression determined by Real Time RT-PCR.
b: Culture supernatants from the cultures were collected and 12(S)-HETE concentration determined by ELISA. The results represent the mean±SD of at least 3 independent experiments using triplicate cultures.

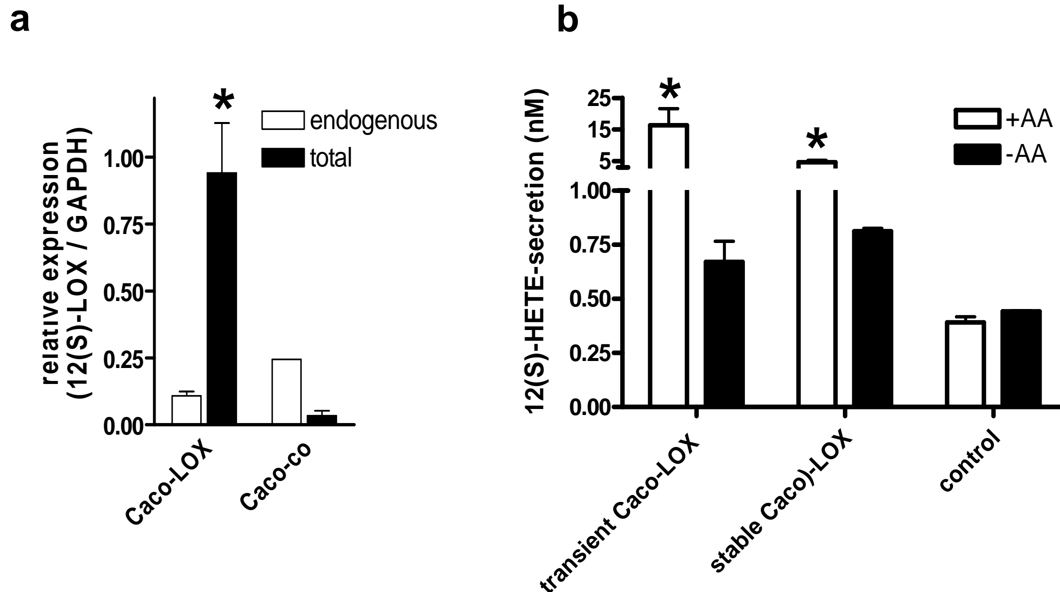

Figure 2s: 12(S)-LOX over expression in Caco2 colon carcinoma cells
Caco2 cells were transfected with a 12(S)-LOX expression vector or a control construct. 24 hours later 10µM AA was added to the medium. (a) RNA was isolated another 24 hours later and 12(S)-LOX expression determined by RT-PCR using primers binding within the coding region of the gene to assess total 12(S)-LOX mRNA (total) or primers binding in the regulatory regions of the gene that were deleted in the expression vector to show endogenous 12(S)-LOX background (endogenous). Band intensity was determined using ImageQuant software (b).
(c) Media were collected from cultures transiently expressing 12(S)-LOX 2 days after transfection and from clone pools stably expressing the enzyme and 12(S)-HETE secretion was determined by ELISA.
All data points represent the mean±SEM of 3 independent experiments using triplicate cultures. * increased above control at $p<0.05$

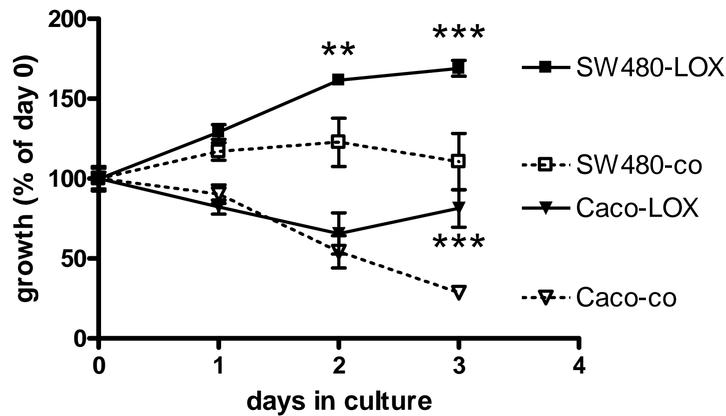

Figure 3s: Impact of 12(S)-LOX over-expression on cell growth

Transfected Caco2 and SW480 cells were grown in the presence of 10µM AA and cell viability determined by neutral red uptake at the indicated time points. All data points represent the mean±SEM of 3 independent experiments using triplicate cultures. ** and *** increased above control at $p<0.01$ and 0.001 respectively.

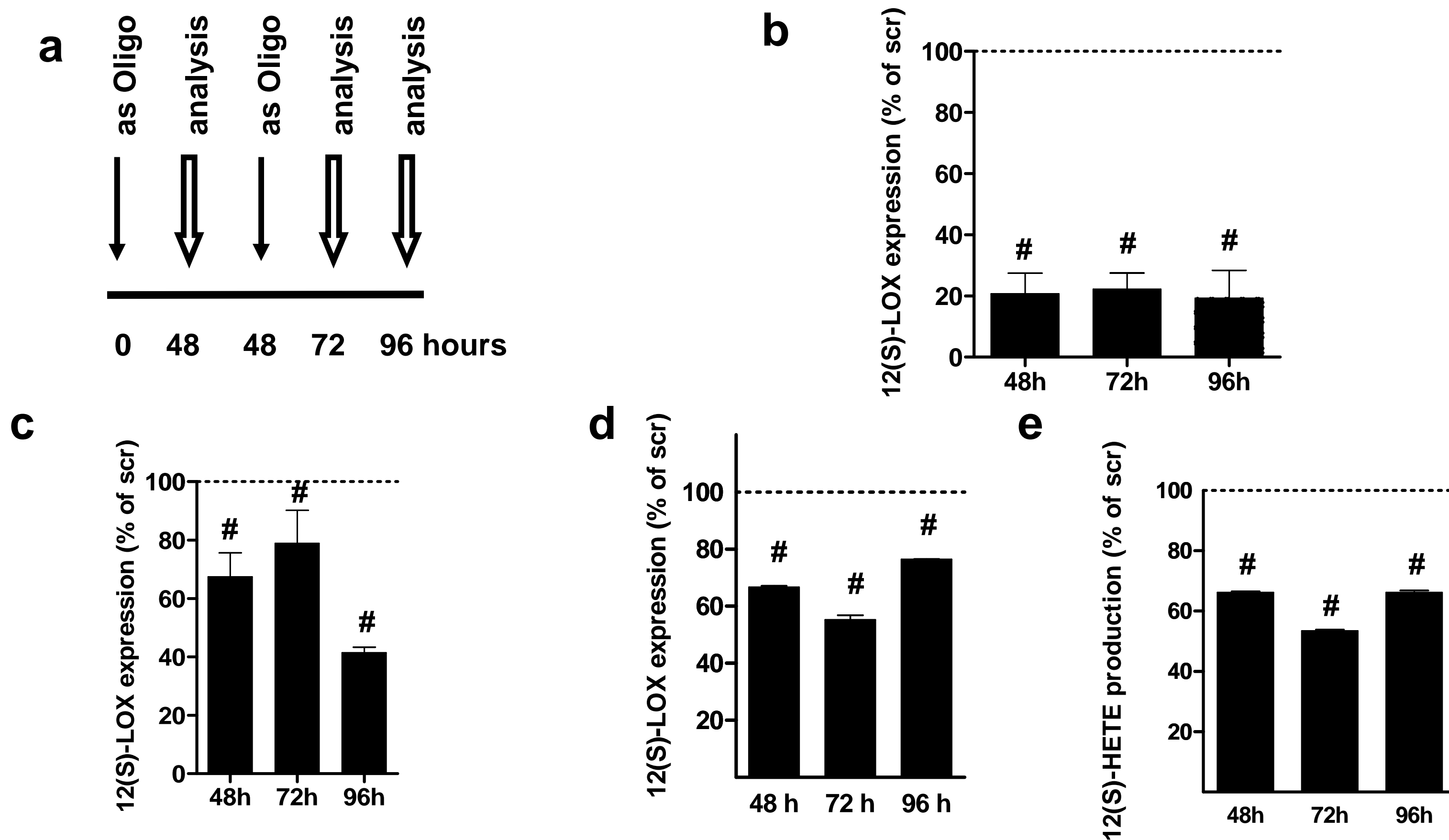

Figure 4s: Knock-down of 12(S)-LOX expression

a: anti-sense nucleotides targeting 12(S)-LOX were introduced into stably 12(S)-LOX over expressing Caco and SW480 cells as well as untransfected SW620 cells and RNA and medium were harvested 48 hours later. Parallel cultures received a second anti-sense dose after 48 hours and another analysis was performed after a total of 72 and 96 hours.

b-d: 12(S)-LOX expression was determined by Real Time RT-PCR from (b) SW620 cells, (c) SW480-LOX and (d) Caco2-LOX cells.

e: To demonstrate down modulation of the enzyme activity 12(S)-HETE was quantified from the culture supernatant.

All results are the mean±SEM of 3 independent experiments using duplicate data points.

decreased above control at $p<0.05$

3. Analysis of gene expression

Micro array analysis

For an initial analysis of gene expression Caco2 cells transiently over-expressing 12(S)-LOX were used because of their high HETE production upon AA addition (about 20nM; see supplemental materials Figure 2s b). RNA was isolated 24 hours after supplementation of the culture medium with 20µM AA and as described above and cDNA was synthesized and labelled with 50µCi [α-^{32}P]-dCTP (GE Healthcare, Piscataway, NJ) in a 50µl reaction mix using the AmpoLabelling-LPR Kit (Super Array Bioscience corporation, Frederick, MD).

The labelled cDNA was hybridized to a GEArray Q Series HS-007 membrane array (Super Array Bioscience corporation, Frederick, MD) containing 96 metastasis associated genes and 4 house keeping genes. The amount of hybridized label was determined by a Phospho-Imager SI (GE Healthcare) and quantified using the GEArray Expression Analysis Suite 1.0 (Super Array Bioscience Corporation). Signals stronger than 2x background that could be detected in all the repeated hybridizations performed were considered to be sufficiently robust for follow-up. They are listed in Table 3s:

RT-PCR

Table 1s: RealTime PCR Assay Kits

Gene code	Product number
ALOX12	Hs00167524_m1
CDH1	Hs00170423_m1
ITGB1	Hs00236976_m1
ITGA5	Hs00233808_m1
GAPDH	Hs99999905_m1

Table 2s: Standard RT-PCR conditions

gene	**primer**	**annealing temperature**
12(S)-LOX in tissue	forward: 5’CATGCTGCAGCAGGAGAGCTGAG-3’	56°C
	reverse: 5’CTACCTGCAAGATTTCACATCA-3’	
12(S)-LOX in over-expressing cells	forward: 5’CTG GAC TTT GAA TGG ACA CT-3’	52°C
	reverse: 5’-GGG AGG CTG AAT CTG GA-3’	
GAPDH	forward: 5'-CGG GAA GCT TGT GAT CAA TGG-3'	53°C
	reverse: 5'-GGC AGT GAT GGC ATG GAC TG-3'	

Table 3s: Down-regulated genes identified by the Metastasis Super Array

Gene code	Gene name	control		12(S)-LOX		Relative expression LOX/co	p<0.05
		mean	*SD*	*mean*	*SD*		
CDH1	E-cadherin	0,078	0,005	0,030	0,010	0,38	#
CST3	Cystatin C	0,088	0,013	0,044	0,016	0,50	#
CTSB	Cathepsin B	0,070	0,004	0,021	0,005	0,29	#
CTSD	Cathepsin D	0,129	0,008	0,102	0,019	0,79	
DCC	Deleted in Colorectal Carcinoma	0,101	0,012	0,070	0,018	0,69	#
ITGA5	Integrin α5	0,153	0,080	0,057	0,024	0,37	#
ITGB1	Integrin β1	0,080	0,003	0,024	0,005	0,30	#
LAMB1	Laminin B1	0,100	0,008	0,044	0,006	0,45	#
LAMC1	Laminin C1	0,095	0,004	0,035	0,001	0,37	#
MGEA5	Meningioma expressed antigen 5	0,088	0,010	0,025	0,004	0,28	#
MMP15	Matrix Metallo-proteinase 15	0,095	0,0001	0,076	0,011	0,80	#
NME1	non-metastatic cells 1	0,136	0,013	0,114	0,020	0,84	
ODC1	Ornithin Decarboxylase	0,094	0,017	0,031	0,008	0,33	#
RAC1	ras-related C3 botulinum toxin substrate 1	0,308	0,048	0,252	0,049	0,82	
RAF1	Ras-associated factor	0,051	0,001	0,025	0,007	0,50	#
TGFB1	Transforming growth factor b1	0,052	0,019	0,065	0,024	1,25	
TIMP2	Tissue inhibitor of metalloproteases	0,125	0,025	0,090	0,011	0,72	