Supplemental Information for:

Transcellular transport of cobalamin in aortic endothelial cells

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Tables

		Radioactivity (cpm)	
		Filtrates	**Filters**
Radioactive Top	**Upper**	49	15974
	Lower	33	4489
Radioactive Bottom	**Upper**	42	4948
	Lower	49	14662
Control: Radioactive Top	**Upper**	47	9433
	Lower	33	615
Control: Radioactive Bottom	**Upper**	48	414
	Lower	37	8120

Table S1. Determination of the amounts of free versus protein-bound [^{57}Co]-Cbl in the upper and receiving chambers. [^{57}Co]-CNCbl-TC was added at the top or bottom compartments of the cell culture transwell system ('radioactive top', 'radioactive bottom'). An aliquot of conditioned medium was taken after time, spun-down using microcentrifuge filters with MWCO 5,000 Da, and the radioactivity of the filtrate and filters was measured. Presence of radioactivity in the filtrate represents free [^{57}Co]-Cbl, whereas retention of radioactivity in the filter indicates that [^{57}Co]-Cbl is bound to protein with a molecular weight larger than 5,000 Da. Regardless of the site of the source radioactive material, transcytosed B_{12} appeared to exit the cell bound to protein, or to bind to apo-protein present in the conditioned medium readily. Two controls were carried out ('Control: Radioactive Top/Bottom'), using transwell inserts without cells, to account for passive diffusion of holo-TC. At the concentrations of [^{57}Co]-CNCbl-TC utilized in the present study (110 pM [^{57}Co]-CNCbl-TC + 33 pM unlabeled holo-TC from 5% FBS, source chamber), full equilibration of concentrations by passive diffusion against medium containing a basal amount of 33 pM holo-TC on the other side of the transwell (receiving chamber), did not occur within the experimental time-frame. Mobilization of [^{57}Co]-Cbl by transcellular transport (transcytosis and exocytosis) appeared to outcompete passive diffusion.

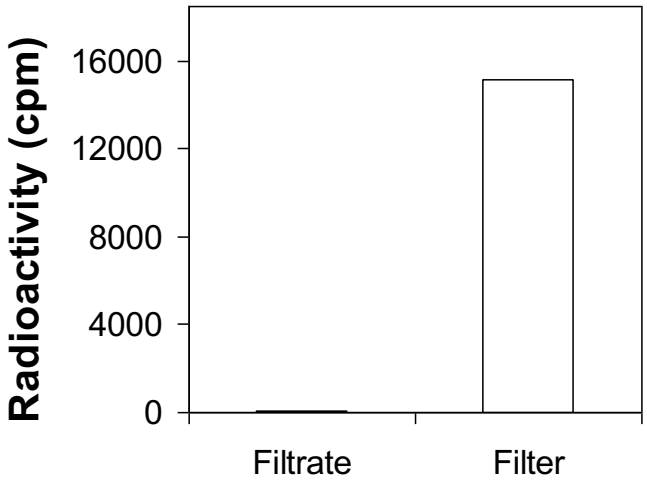
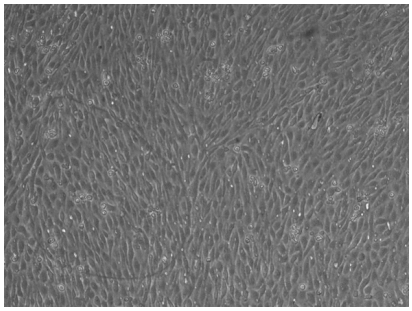
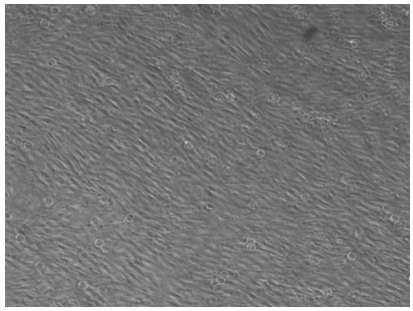


Figure S1. Quality of the [^{57}Co]-CNCbl-TC preparation for transcytosis experiments. [^{57}Co]-CNCbl (110 pM) was incubated with 4-fold excess apo-TC for 16 h at 37°C, in Ham's/F12 supplemented with 5% FBS and 200 nM *N*^{5}-MeTHF. An aliquot was transferred to a 5,000 Da cut-off spin filter and the radioactivity associated with the filter and the filtrate determined. Approximately 99.6% of [^{57}Co]-CNCbl was bound to TC. All experiments were performed with [^{57}Co]-holo-TC prepared by this method.

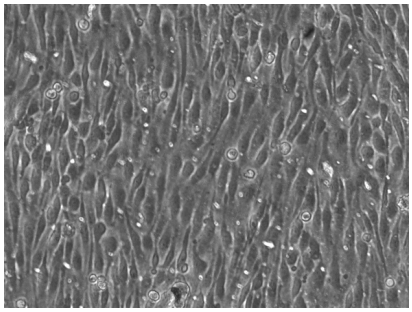
BAEC, 10X



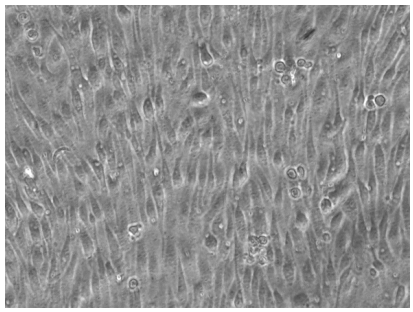
BAEC 10X, (edge)



BAEC, 20X



BAEC 20X, (edge)



BAEC 40X

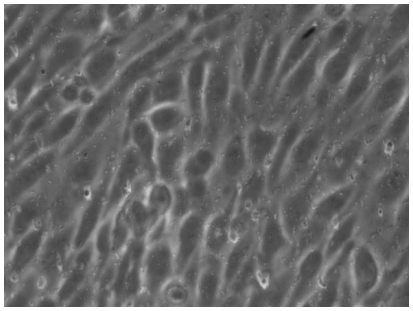


Figure S2. Phase-contrast microscopy of confluent BAEC grown on transwells (PET, coated with Collagen I, BD Biosciences). Photographs were taken at the center and at the edges of the transwells. Images shown at 10X, 20X and 40X magnification.

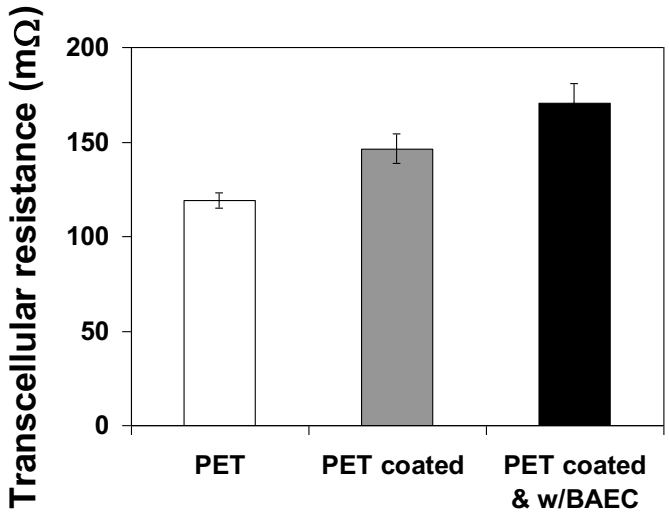


Figure S3. Transcellular resistance measurements in confluent monolayers of BAEC. Measurements were carried out with an Ag/AgCl electrode (Millipore Millicell® -ERS). An increase of 20 mΩ in PET-coated & w/BAEC (n=31) or higher compared to PET (n=8) and PET-coated (n=15) was considered acceptable for further experimentation. These results are in agreement with values of resistance (mΩ) provided by the manufacturer of the PET transwell systems (BD Biosciences).

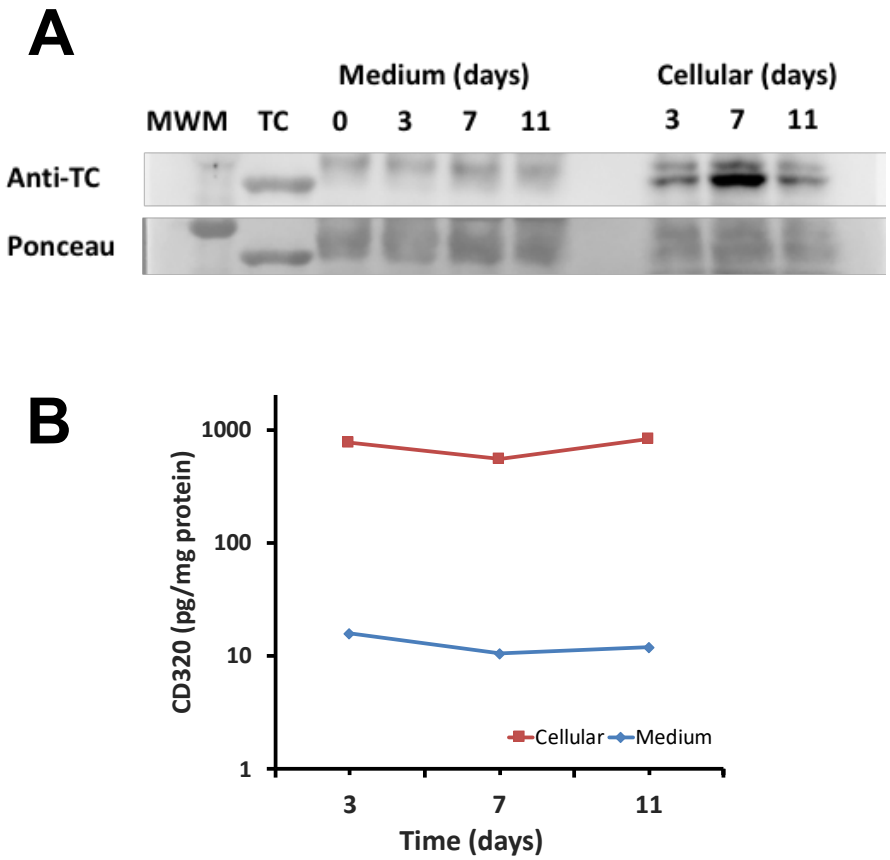


Figure S4. Expression of TC and CD320 by BAEC. A. Western blot of TC in conditioned medium (undiluted) and whole cell extracts of BAEC using a polyclonal human anti-TC (abcam 189871, dilution 1:500). Maximum detection of TC was observed in confluent whole cell extracts of BAEC. Ponceau staining of the western blot membrane is shown as a control of protein transfer efficiency and relative loading amounts. The molecular weight marker (MWM) band revealed with Ponceau staining corresponds to 55 kDa. The molecular weight of bovine TC is 47 kDa. Pure bovine recombinant TC (10 µg) was loaded as control (well labelled as 'TC'). **B.** ELISA detection of CD320 receptor (Abcam product # ab213759) examined in conditioned culture medium (undiluted) and in whole cell extracts of BAEC at 3, 7 and 11 days. At time zero, the total amount of CD320 in conditioned culture medium was 11.5 ± 0.2 pg/mg protein. Our data suggests that very low levels of soluble CD320 are secreted into the culture medium.

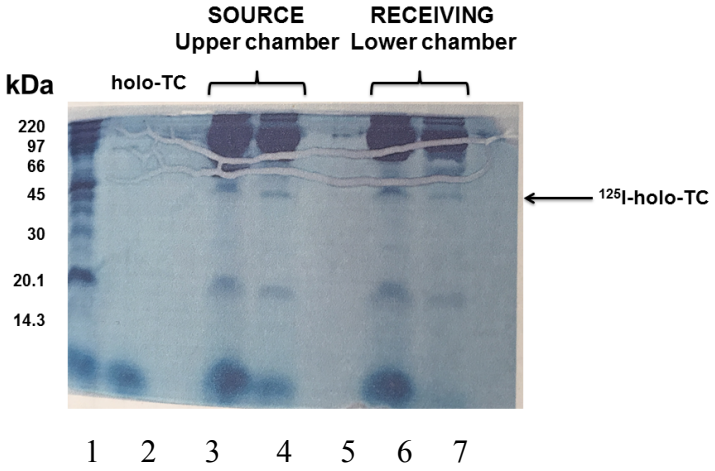


Figure S5. Gel electrophoresis under native conditions (without SDS) of aliquots of conditioned culture medium in source and receiving chambers of transwell inserts. From left to right: lane 1: molecular weight marker (^{14}C-methylated protein Rainbow marker, Amersham); lane 2: control ^{125}I-holo-TC (2 μg); lane 3: empty; lanes 4 and 5: 35 and 20 μL of conditioned medium from source chamber; lanes 6 and 7: empty; lanes 8 and 9: 35 and 20 μL of conditioned medium from receiving chamber (the expected band for transcytosed ^{125}I-holo-TC is shown with an arrow); lane 10: empty. The gel was stained with Coomassie brilliant blue R-250. This gel was analyzed by phosphorimaging, as shown in Figure S6.

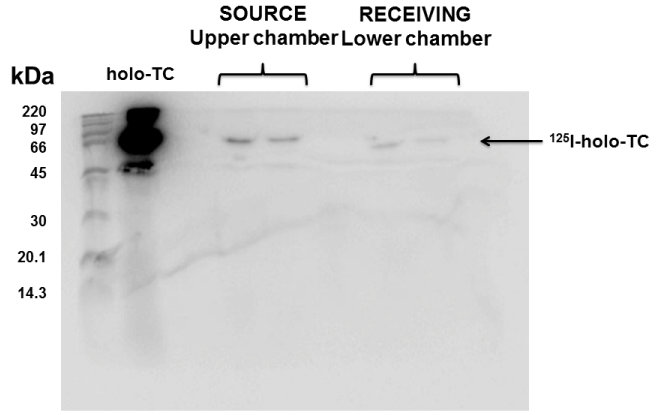


Figure S6. Fate of ^{125}I-holo-TC in endothelial cells. Labeling of CNCbl-TC with ^{125}I permitted the monitoring of CNCbl-TC transport across the endothelial monolayer. After 24 h, approximately 40% of the internalized ^{125}I-holo-TC had decomposed to peptides of molecular weight lower than 5,000 Da (Amicon filtrate, not loaded on the gel). This represents the pool of holo-TC that furnishes intracellular processing and delivery of Cbl to MS and MCM. Phosphorimager of the protein-bound fraction in the source and receiving chambers showed that intact ^{125}I-holo-TC is also present in the receiving chamber. This pool of holo-TC represents a fraction of the source ^{125}I-holo-TC that bypassed the lysosomal degradation system.