# Supplemental Information

## Serotonin biosynthesis as a predictive marker of serotonin pharmacodynamics and disease-induced dysregulation

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## Supplementary methods

### qPCR analysis of gene expression in the bleomycin model

The 69 gene panel was made up of 9 genes related to 5-HT, 26 genes for extracellular matrix synthesis and degradation, 12 genes from an idiopathic pulmonary fibrosis translational panel[1] and 22 genes from other fibrotic processes. Total RNA from the lung of the rats was isolated using the RNeasy mini kit according to the manufacturer's protocol (QIAGEN, Germany). Remaining genomic DNA was digested using the DNAse free kit (Ambion, USA). The quantity of RNA was analyzed using a Nanodrop spectrophotometer (Thermo Fisher, USA) and RNA quality was assessed using a Bioanalyzer 2100 (Agilent, USA). 1000 ng total RNA was reverse transcribed with the high capacity cDNA archive kit according to the manufacturer's protocol (Applied Biosystems, USA). The cDNA was preamplified according to the preamp kit with 14 cycles (Applied Biosystems, USA). QPCR was performed on an Biomark HD (Fluidigm, USA) using 96*96 dynamic arrays using Taqman assays **(Supplementary Table 2)** (Applied Biosystems, USA). Based on GENORM [2] 28s-Mm03682676_s1, Hprt1-Rn01527840_m1 and Pgk1-Rn00821429_g1 were used as reference genes. Expression values were calculated using a modified delta Ct method where an expression value of 1 reflects no detectable expression.

### Quantitation of TPH inhibitors in plasma

PCPA, LX-1032 and LX-1032 metabolite were quantified by LC-MS/MS. Standard curves were generated in blank plasma (0.51-10,000 ng/mL). Samples were analyzed by a narrow-bore liquid chromatography method. A Luna® C8, 5 μm, 20 x 2.0 mm (Phenomenex®) column was used at a flow rate of 0.6 mL/min with mobile phase A: 0.1 % aqueous formic acid and mobile phase B: acetonitrile. A 0.15 minute isocratic step of 5% mobile phase B was followed by a 0.45 minute (0.25 minute for PCPA) gradient from 5% to 95% mobile phase B. Finally the column was cleaned and re-equilibrated. An API-4000 (ABSciex™) and an API 5000 (ABSciex™) Triple Quadrupole LC-MS/MS Mass Spectrometer, both equipped with a Turbo spray, were used for PCPA and LX-1032 (LX-1032 metabolite), respectively.

For PCPA, The plasma samples from the 30 mg/kg dose and the 100 mg/kg dose were diluted with blank plasma 30 and 100 times, respectively. Calibrant and samples were protein precipitated by addition of 3 volumes of methanol containing phenylalanine as an internal standard, cleared by centrifugation and diluted (1:1, v/v) with mobile phase. A 15 μL volume was injected on the LC-MS/MS.

For LX-1032 and LX-1032 metabolite, calibrant and plasma samples were stabilized with 0.1% dichlorvos (v/v) and diluted 3 times with blank plasma. Calibrant and samples were protein precipitated by addition of 3 volumes of methanol containing LX1031 as an internal standard, cleared by centrifugation and diluted (1:1, v/v) with mobile phase. A 3 μL volume was injected on the LC-MS/MS.

LC-MS/MS was run with positive ion electrospray. The following MRM transitions (Q1/Q3) were used for quantitation of PCPA (199.9/154.1), LX-1032 (575/273.2) and LX1032

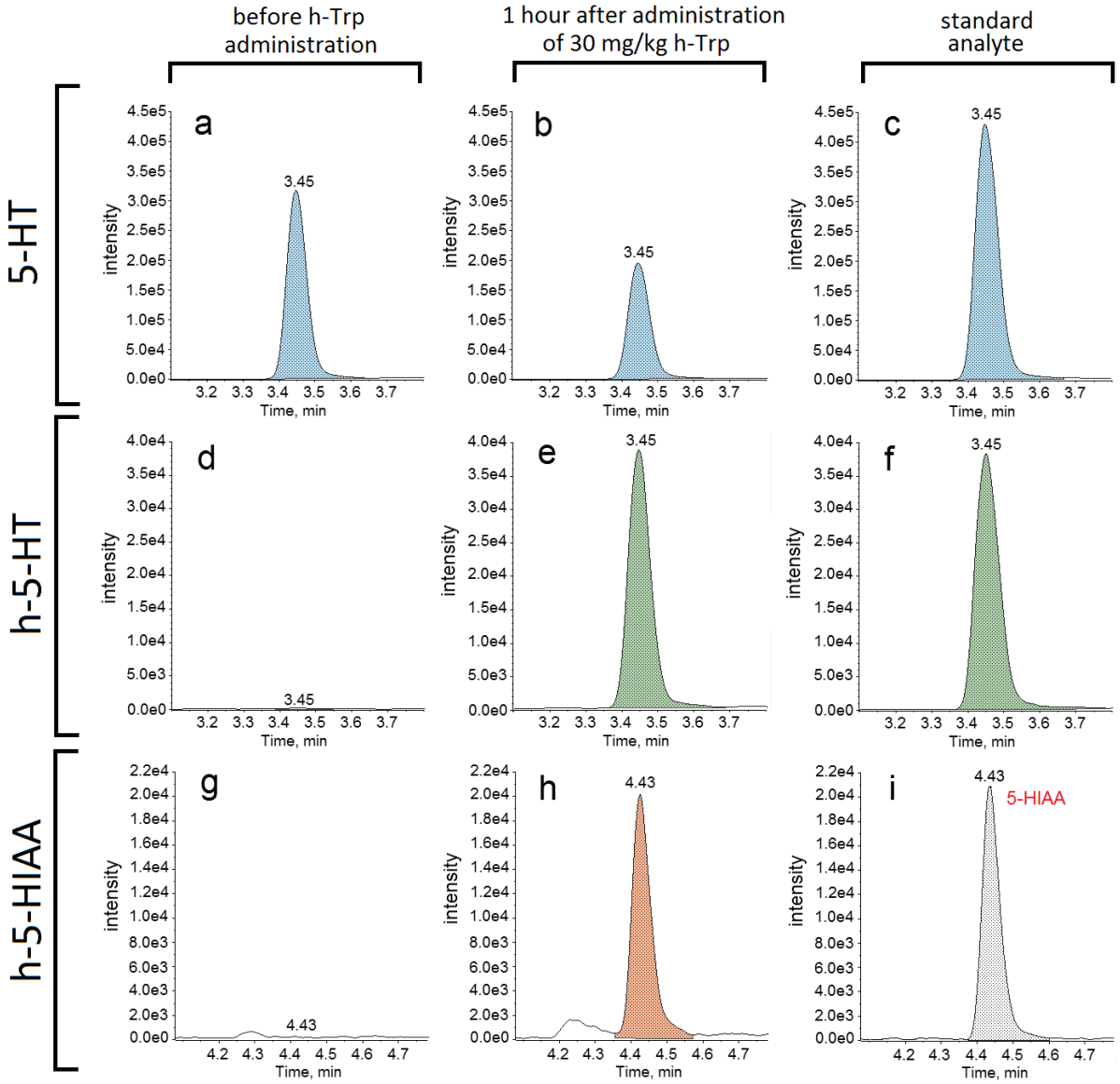
metabolite (547.1/272.9). Pharmacokinetic parameters were estimated using the Phoenix software package (Pharsight Corporation, Cary, NC, USA) using non-compartmental analysis and the linear up/ log trapezoidal down calculation method and without weighing. Calculated terms were defined as follows; $C_{max}$, maximum observed plasma concentration; $T_{max}$, time of maximum observed plasma concentration; $AUC_{0\text{-}last}$, area under the plasma concentration vs. time curve up to the last measurable concentration, calculated by the log-linear trapezoidal rule.

**BON cell assay**

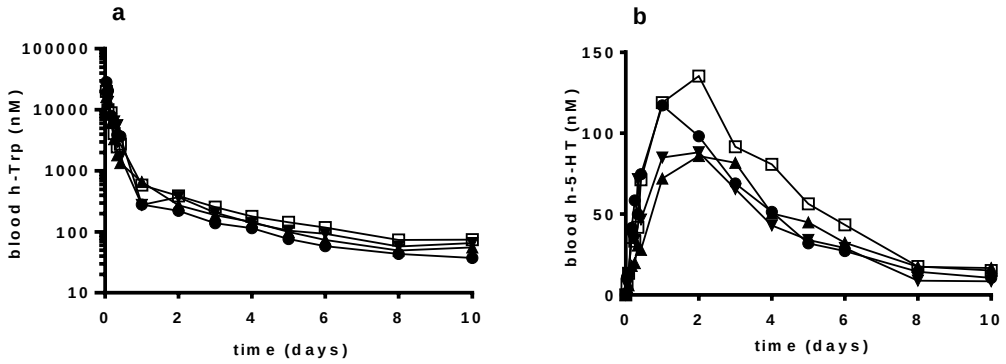
The human pancreatic carcinoid cell line BON expressing *tph1* endogenously and secreting 5-HT [3] was obtained from Prof. Dr. Gudrun Ahnert-Hilger (Charité, Berlin). The cells were seeded at a density of 100000 cells/well in a 96-well plate and incubated in complete growth medium overnight at 37°C, 5% $CO_2$. For the treatment with TPH inhibitors, the full growth medium was replaced with serum free medium. Cells were treated with TPH inhibitors at 12 concentrations for 16 hours. Supernatants were collected and passed through a Multiscreen+HiFlow glass fiber FB plate (Millipore) under vacuum. The samples were dried in a SpeedVac (Concentrator plus, Eppendorf), resuspended in 0.1M acetic acid/ sodium actetate at pH 3.5 (buffer A), separated on a Atlantis T3 column (Waters, #186003726) using high pressure liquid chromatography (Dionex UltiMate 3000 RSLC) running a 2.5 minute gradient from 95% to 10% buffer A at 1.5mL/min (buffer B is acetonitrile). 5-hydroxytryptophan (5-HTP) was quantified with a Dionex UltiMate 3000 FLD-3400RS fluorescence detector $\lambda_{ex}$ = 280nM and $\lambda_{em}$ = 330nm. Inhibitory activities of compounds were determined by calculating the $IC_{50}$ value i.e. the concentration of compound needed to inhibit 50% of cellular 5-HTP formation.

## Supplementary figures and tables

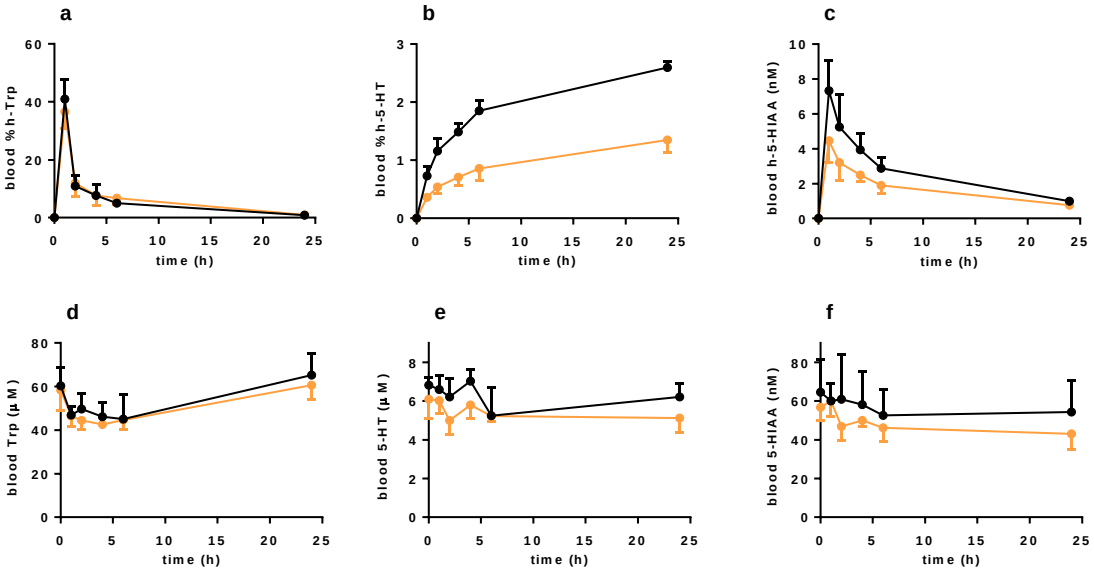
**Supplementary Figure 1:** Chromatograms of 5-HT, h-5-HT and h-5-HIAA in rat blood demonstrate method specificity. (Figure **(a)** to Figure **(i)**) show the MRM (/Q3) chromatograms (intensity vs. elution time (min)) generated by LC-MS/MS of 5-HT (178/161) (Figure **(a)** to Figure **(c)**), h-5-HT (171/142.1) (Figure **(d)** to Figure **(f)**) and h-5-HIAA (203/156) (Figure **(g)** and **(h)**) and 5-HIAA (Figure **(i)**). **(a)**, **(d)** and **(g)** are measured from a blood sample from a rat before h-Trp administration. **(b)**, **(e)** and **(h)** are measured from a blood sample from the same rat 1 hour after administration of 30 mg/kg h-Trp. **(c)**, **(f)** and **(i)** are the chromatograms of standards (note no standard of h-5-HIAA was available, however, it co-elutes with the naturally occurring 5-HIAA, see **(i)**).



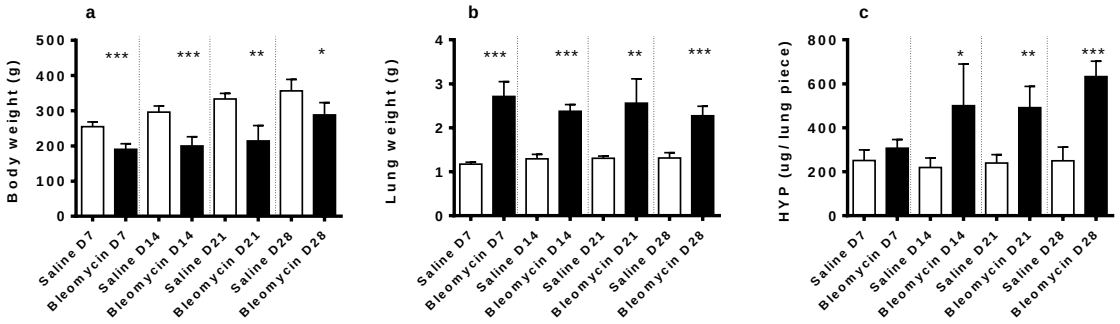
**Supplemental Figure 2**: Wash-out of stable isotope labeled molecules from rat blood. **(a)** h-Trp and **(b)** h-5-HT. At time 0, rats were given an oral dose of 40 mg/kg h-Trp and serial blood samples were taken. Each trace represents an individual rat (n = 4). For h-5-HT the half-life quoted in the text was generated by fitting the data between 2 and 10 days to a single exponential decay.



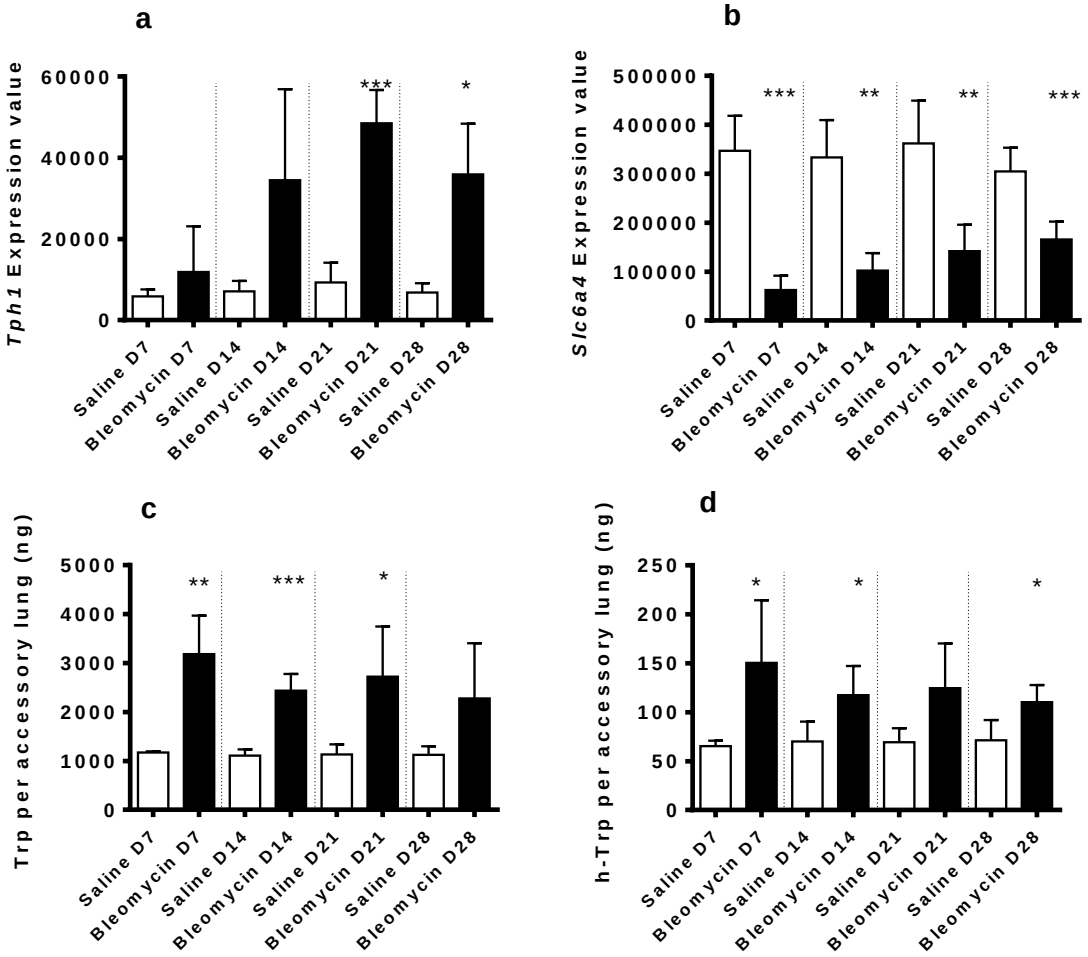
**Supplemental Figure 3:** Inhibitory effects of a single dose of LX-1032 on conversion of orally administered h-Trp to h-5-HT. **(a)** Blood %-h-Trp; **(b)** blood %h-5-HT; **(c)** blood h-5-HIAA; **(d)** blood Trp; **(e)** blood 5-HT; **(f)** blood 5-HIAA. Solid black circles are vehicle, solid orange circles were pretreated orally with 23 mg/kg LX-1032, 1 hour prior to oral h-Trp administration (n = 4, ± SD).



**Supplemental Figure 4:** Disease induction 7, 14, 21 and 28 days after bleomycin administration in the lung fibrosis model. Measurement at sacrifice of **(a)** Body weight, **(b)** lung weight and **(c)** lung hydroxyproline (HYP) in acid hydrolyzate as a collagen surrogate (n = 4-6 ± SD). A two tailed student's t-test comparing bleomycin to NaCl for individual time points is given; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.



**Supplemental Figure 5**: Gene expression and metabolite changes of the 5-HT pathway from the lung in the bleomycin lung fibrosis model. **(a)** *Tph1* mRNA; **(b)** *Slc6a4* (*Sert*) mRNA. Additionally, Trp **(c)** and h-Trp **(d)** levels in lung are shown (n = 4-6 ± SD). A two tailed student's t-test comparing bleomycin to NaCl for individual time points is given; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.



**Supplementary Table 1:** $IC_{50}$ values and pharmacokinetics of the TPH inhibitors tested for their ability to inhibit production of h-5-HT after the acute administration of h-Trp to rats. Shown are mean $IC_{50}$ values for inhibition of 5-HTP synthesis in BON cells after 16 hours incubation in serum free assay medium. Additionally, pharmacokinetic parameters for the experiments in Figure 2 are given ($AUC_{0\text{-last}}$ and $C_{max}$ as geometric means, $t_{max}$ as median). For 6, 20 and 60 mg/kg of LX-1032, the final timepoint was 10.5 hours after dosing (n = 5-6). For PCPA, the final time point was 25 hours after dosing (n = 4). For LX-1032, values are provided for both the administered ester and the corresponding acid metabolite in square parenthesis.

| **Compound** | **Dose** | **BON $IC_{50}$ (nM)** | **$AUC_{0\text{-last}}$ (h*ng/mL)** | **$C_{max}$ (ng/mL)** | **$t_{max}$ (h)** |
|---|---|---|---|---|---|
| PCPA | 30 | 1965 (n=2) | 315000 | 25200 | 3 |
| PCPA | 100 | 1965 (n=2) | 1370000 | 81500 | 5 |
| LX-1032<br>[metabolite] | 6 | 12 (n=15)<br>[ 43 (n=2)] | 26<br>[365] | 45<br>[327] | 0.5<br>[0.5] |
| LX-1032<br>[metabolite] | 20 | 12 (n=15)<br>[ 43 (n=2)] | 24<br>[2380] | 27<br>[842] | 1.5<br>[1.3] |
| LX-1032<br>[metabolite] | 60 | 12 (n=15)<br>[ 43 (n=2)] | 78<br>[2470] | 117<br>[1570] | 0.8<br>[0.8] |

**Supplementary Table 2:** Genes measured in qPCR panel.

| Gene | Assay ID |
|---|---|
| *Acta2* | Rn01759928_g1 |
| *Aqp3* | Rn01439528_g1 |
| *Bcl2* | Rn99999125_m1 |
| *C6* | Rn00598544_m1 |
| *Calm1* | Rn00821407_g1 |
| *Ccl1* | Rn01752376_m1 |
| *Ccl2* | Rn01456716_g1 |
| *Cdh1* | Rn00580109_m1 |
| *Cma1* | rn00565319_m1 |
| *Col18a1* | Rn01428995_m1 |
| *Col1a1* | Rn01463848_m1 |
| *Col3a1* | Rn01437681_m1 |
| *Col5a1* | Rn00593170_m1 |
| *Ctgf* | Rn00573960_g1 |
| *Cthrc1* | Rn00597305_m1 |
| *Ctps* | Rn00516751_m1 |
| *Ctse* | Rn01483642_m1 |
| *Cxcl2* | Rn00586403_m1 |
| *Ddc* | Rn00561113_m1 |
| *Ednrb* | Rn00569139_m1 |
| *Fhl2* | Rn00581565_m1 |
| *Fn1* | Rn00569575_m1 |
| *Gal* | Rn01234339_m1 |
| *Grem1* | Rn01509832_m1 |
| *Gsn* | Rn01438922_m1 |
| *Hbegf* | Rn01405658_m1 |
| *Hmox1* | Rn01536933_m1 |
| *Htr1a* | Rn00561409_s1 |
| *Htr1b* | Rn01637747_s1 |
| *Htr2a* | Rn00568473_m1 |
| *Htr2b* | Rn00568450_m1 |
| *Id2* | Rn01495280_m1 |
| *Igfbp7* | Rn01413246_m1 |
| *Il10* | Rn00563409_m1 |
| *Lcn2* | Rn00590612_m1 |
| *Lox* | Rn00566984_m1 |
| *Loxl1* | Rn01418038_m1 |
| *Loxl2* | Rn01466080_m1 |
| *Maoa* | Rn01430950_m1 |
| *Maob* | Rn00566203_m1 |
| *Mmp12* | Rn00588640_m1 |
| *Mmp14* | Rn00579172_m1 |

| | | |
|---|---|---|
| | *Mmp2* | Rn01538170_m1 |
| | *Mmp7* | Rn00689241_m1 |
| | *Nell1* | Rn00675924_m1 |
| | *Nfe2l2* | Rn00477784_m1 |
| | *Pcsk1* | Rn00567266_m1 |
| | *Pde4a* | Rn00565354_m1 |
| | *Pla2g2a* | Rn00580999_m1 |
| | *Pld2* | Rn00583683_m1 |
| | *Ptger2* | Rn00579419_m1 |
| | *Ptges* | Rn00572047_m1 |
| | *Rac1* | Rn01412766_m1 |
| | *Serpine1* | Rn00561717_m1 |
| | *Slc2a5* | Rn00582000_m1 |
| | *Slc6a4* | Rn00564737_m1 |
| | *Smad6* | Rn01766978_m1 |
| | *Sod1* | Rn00566938_m1 |
| | *Spp1* | Rn00681031_m1 |
| | *Tgfb1* | Rn00572010_m1 |
| | *Tgm2* | Rn00571440_m1 |
| | *Thbs2* | Rn02111874_s1 |
| | *Timp1* | Rn01430873_g1 |
| | *Timp2* | Rn0573232_m1 |
| | *Tnc* | Rn01454947_m1 |
| | *Tph1* | Rn01476867_m1 |
| | *Vcan* | Rn01493763_m1 |
| | *Vegfa* | Rn01511601_m1 |
| | *Wisp1* | Rn00581854_m1 |
| control | *28s* | Mm03682676_s1 |
| control | *Actb* | Rn00667869_m1 |
| control | *B2m* | Rn00560865_m1 |
| control | *Gusb* | Rn00566655_m1 |
| control | *Hprt1* | Rn01527840_m1 |
| control | *Pgk1* | Rn00821429_g1 |
| control | *Ppia* | Rn00690933_m1 |

## References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3 Tran, V. S. *et al.* Serotonin secretion by human carcinoid BON cells. *Ann N Y Acad Sci* **1014**, 179-188 (2004).