MicroRNA-23a mediates post-transcriptional regulation of CXCL12 in bone marrow stromal cells

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Supplementary Methods

Culture conditions for cell lines

The KG1a and SCP-1 were cultured in MEMalpha supplemented with 20% fetal calf serum (FCS) (Gibco, Life Technologies, Carlsbad, CA, USA, and 1% Penicillin/Streptomycin (PAA Laboratories, Pasching, Austria). The HS27, HL60, K562, and HeLa cell lines were cultured in RPMI supplemented with 10% FCS and 1% Penicillin/Streptomycin. The HS5 cell line was cultured in DMEM supplemented with 10% FCS and 1% Penicillin/Streptomycin.

Isolation and culture of human BMSCs (hBMSCs)

Bone marrow (BM) was isolated from the iliac crest of patients with myelodysplastic syndrome (MDS) and healthy donors. BM aspirates were mixed with PBS (1:3) and were overlayed on 15 ml Ficoll solution (GE Healthcare, Uppsala, Sweden) in a 50-ml conical tube and centrifuged at 700g for 20 min. Mononuclear cells were collected from the middle layer and interface. The cells were then washed with PBS, resuspended in culture medium, seeded in culture flasks, and incubated at 37°C. After 3–4 days of culture, the culture medium and non-adherent cells were discarded and fresh medium was added every 3 days. After approximately 2 weeks of culture, cells were harvested by trypsinization, and were either frozen or replated for additional experiments. Harvested cells were tested for typical immunophenotypes ($CD73^+$, $CD166^+$, $CD146^+$, $CD105^+$, $CD90^+$, $CD34^-$, and $CD45^-$) by flow cytometry. Phycoerythrin (PE)-conjugated CD34, CD73, and CD166 antibodies and fluorescein isothiocyanate (FITC)-conjugated CD45, CD146, CD105, and CD90 (BD Biosciences, Palo Alto, CA, USA) were used along with the corresponding isotype controls (Beckman Coulter Immunotech, Marseille, France). All studies with human material were approved by the Institutional Review Board of the Medical Faculty of the Dresden University of Technology, and informed consent was obtained from the donors.

Isolation of HSPCs from cord blood and bone marrow

HSPCs were isolated using immunomagnetic selection from cord blood (CB) or peripheral blood of G-CSF-mobilized healthy donors following the manufacturer's instructions (CD34 MicroBead kit; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). After isolation and prior to transwell-migration assays, HSPCs were cultured for 24 h in CellGro^{TM} stem cell growth medium (SCGM; CellGenix, Freiburg, Germany) supplemented with 150 ng/ml FLT3L (Biosource, Camarillo, CA), 150 ng/ml SCF (Biosource) and 50 ng/ml interleukin 3 (Strathmann Biotech, Hannover, Germany).

RNA/miRNA preparation and quantitative RT-PCR

Total RNA was isolated from cultured cells using the mirVana miRNA isolation kit (Ambion Inc, Austin, USA), according to the manufacturer's instructions. Ready-to-use RNA from FirstChoice® Human Total RNA Survey Panel (Ambion) was used for tissue determination of miRNA expression. cDNA was generated by reverse transcription using SuperScript II. Real-time RT-PCR was performed using a 7500 Real-Time PCR system (Applied Biosystems, Foster city, CA, USA). Validated PCR primers and TaqMan probes for CXCL12 were used. Thermal cycler conditions for TaqMan primers were as follows: 2 min 50°C; 10 min 95°C; for 40 cycles, denaturation (15 s, 95°C) and combined annealing/extension (1 min, 60°C).

The expression levels of mature miRNAs were quantified by RT-PCR using TaqMan primers. Reverse transcription was performed using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems). U6 snRNA (001973) served as an internal reference.

The mRNA or miRNA levels were normalized against housekeeping genes (GAPDH and U6, respectively) and relative target gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method.

Supplementary Table 1

No.	Pre-miRNA
	pre-miR control 30pmol
	siSDF1 30pmol
1	pre-let-7a
2	pre-let-7b
3	pre-let-7c
4	pre-let-7d
5	pre-let-7e
6	pre-let-7f
7	pre-let-7g
8	pre-let-7i
9	pre-miR-1a
10	pre-miR-100
11	pre-miR-101
12	pre-miR-103
13	pre-miR-105
14	pre-miR-106a
15	pre-miR-106b
16	pre-miR-107
17	pre-miR-10a
18	pre-miR-10b
19	pre-miR-124a
20	pre-miR-125a
21	pre-miR-125b
22	pre-miR-126
23	pre-miR-126*
24	pre-miR-127
25	pre-miR-128/128a
26	pre-miR-128/128b
27	pre-miR-129
28	pre-miR-130a
29	pre-miR-130b
30	pre-miR-132
31	pre-miR-133a
32	pre-miR-133b
33	pre-miR-134
34	pre-miR-135a
35	pre-nmiR-135b
36	pre-miR-136
37	pre-miR- 137
38	pre-miR-138
39	pre-miR-139
40	pre-miR-140
41	pre-miR-141
42	pre-miR-142-3p
43	pre-miR-142-5p
44	pre-miR-143
45	pre-miR-144
46	pre-miR-145
47	pre-miR-146a
48	pre-miR-146b
49	pre-miR-147
50	pre-miR-148a
51	pre-miR-148b-3p
52	pre-miR-149
53	pre-miR-150
54	pre-miR-151
55	pre-miR-152
56	pre-miR-153
57	pre-miR-154
58	pre-miR-154*
59	pre-miR-155
60	pre-miR-15a
61	pre-miR-15b
62	pre-miR-16
63	pre-miR-17-3p
64	pre-miR-17-5p
65	pre-miR-181a
66	pre-miR-181a*
67	pre-miR-181b
68	pre-miR-181c
69	pre-miR-181d
70	pre-miR-182
71	pre-miR-182*
72	pre-miR-183
73	pre-miR-184
74	pre-miR-185
75	pre-miR-186
76	pre-miR-187
77	pre-miR-188
78	pre-miR-189
79	pre-miR-18a
80	pre-miR-18a*

81	pre-miR-18b	121	pre-miR-219
82	pre-miR-190	122	pre-miR-22
83	pre-miR-191	123	pre-miR-220
84	pre-miR-191*	124	pre-miR-221
85	pre-miR-192	125	pre-miR-222a
86	pre-miR-193	126	pre-miR-223
87	pre-miR-193b	127	pre-miR-224
88	pre-miR-194	128	pre-miR-23a
89	pre-miR-195	129	pre-miR-23b
90	pre-miR-196a	130	pre-miR-24
91	pre-miR-196b	131	pre-miR-25
92	pre-miR-197	132	pre-miR-26a
93	pre-miR-198	133	pre-miR-26b
94	pre-miR-199a	134	pre-miR-27a
95	pre-miR-199*	135	pre-miR-27b
96	pre-miR-199b	136	pre-miR-28
97	pre-miR-19a	137	pre-miR-296-5p
98	pre-miR-19b	138	pre-miR-299-3p
99	pre-miR-200a	139	pre-miR-299-5p/299*
100	pre-miR-200a*	140	pre-miR-29a
101	pre-miR-200b	141	pre-miR-29b
102	pre-miR-200c	142	pre-miR-29c
103	pre-miR-202	143	pre-miR-301
104	pre-miR-202*	144	pre-miR-302a
105	pre-miR-203	145	pre-miR-302a*
106	pre-miR-204	146	pre-miR-302b
107	pre-miR-205	147	pre-miR-302b*
108	pre-miR-206	148	pre-miR-302c
109	pre-miR-208	149	pre-miR-302c*
110	pre-miR- 20a	150	pre-miR-302d
111	pre-miR- 20b	151	pre-miR-30a*/30a-3p
112	pre-miR-21	152	pre-miR-30a/30a-5p
113	pre-miR-210	153	pre-miR-30b
114	pre-miR-211	154	pre-miR-30c
115	pre-miR-212	155	pre-miR-30d
116	pre-miR-214	156	pre-miR-30e*
117	pre-miR-215	157	pre-miR-30e
118	pre-miR-216	158	pre-miR-31
119	pre-miR-217	159	pre-miR-32
120	pre-miR-218	160	pre-miR-320

161	pre-miR-323	201	pre-miR-376b
162	pre-miR-324-3p	202	pre-miR-377
163	pre-miR-324-5p	203	pre-miR-378*
164	pre-miR-325	204	pre-miR-379
165	pre-miR-326	205	pre-miR-380
166	pre-miR-328	206	pre-miR-380*/380-5p
167	pre-miR-329	207	pre-miR-381
168	pre-miR-33	208	pre-miR-382
169	pre-miR-330-3p	209	pre-miR-383
170	pre-miR-331	210	pre-miR-384
171	pre-miR-335/335-5p	211	pre-miR-409-3p
172	pre-miR-337	212	pre-miR-409-5p
173	pre-miR-338	213	pre-miR-410
174	pre-miR-339	214	pre-miR-411
175	pre-miR-33b	215	pre-miR-412
176	pre-miR-340-3p	216	pre-miR-421
177	pre-miR-342	217	pre-miR-422a
178	pre-miR-345	218	pre-miR-422b
179	pre-miR-346	219	pre-miR-423
180	pre-miR-34a	220	pre-miR-424
181	pre-miR-34b*	221	pre-miR-425*
182	pre-miR-34c	222	pre-miR-425-5p
183	pre-miR-361	223	pre-miR-429
184	pre-miR-362-5p	224	pre-miR-431
185	pre-miR-363	225	pre-miR-432
186	pre-miR-363*	226	pre-miR-432*
187	pre-miR-365	227	pre-miR-433
188	pre-miR-367	228	pre-miR-448
189	pre-miR-368	229	pre-miR-449a
190	pre-miR-369-3p	230	pre-miR-449b
191	pre-miR-369-5p	231	pre-miR-450
192	pre-miR-370	232	pre-miR-451
193	pre-miR-371	233	pre-miR-452
194	pre-miR-372	234	pre-miR-452*
195	pre-miR-373	235	pre-miR-453
196	pre-miR-373*	236	pre-miR-454-3p
197	pre-miR-374	237	pre-miR-454*
198	pre-miR-375	238	pre-miR-455-5p/456*
199	pre-miR-376a	239	pre-miR-483
200	pre-miR-376a*	240	pre-miR-484

241	pre-miR-485-3p
242	pre-miR-485-5p
243	pre-miR-486/486-5p
244	pre-miR-487a
245	pre-miR-487b
246	pre-miR-488*
247	pre-miR-489
248	pre-miR-490
249	pre-miR-491
250	pre-miR-492
251	pre-miR-493
252	pre-miR-493*
253	pre-miR-494
254	pre-miR-495
255	pre-miR-496
256	pre-miR-497
257	pre-miR-498
258	pre-miR-499
259	pre-miR-500*
260	pre-miR-501-5p
261	pre-miR-502-5p
262	pre-miR-503
263	pre-miR-504
264	pre-miR- 505
265	pre-miR-506
266	pre-miR- 507
267	pre-miR- 508-3p
268	pre-miR- 509
269	pre-miR- 510
270	pre-miR- 511
271	pre-miR- 512-3p
272	pre-miR- 512-5p
273	pre-miR- 513a
274	pre-miR- 514
275	pre-miR- 515-3p
276	pre-miR- 515-5p
277	pre-miR- 516a-3p/516b*
278	pre-miR- 516a-5p
279	pre-miR- 517*
280	pre-miR- 517a
281	pre-miR- 517b
282	pre-miR- 517c
283	pre-miR- 518a
284	pre-miR- 518b
285	pre-miR- 518c
286	pre-miR- 518c*
287	pre-miR- 518d-3p
288	pre-miR- 518e
289	pre-miR- 518f
290	pre-miR- 518f*
291	pre-miR- 519a
292	pre-miR- 519b
293	pre-miR- 519c-3p
294	pre-miR- 519d
295	pre-miR- 519e
296	pre-miR- 519e*
297	pre-miR- 520a-3p
298	pre-miR- 520a-5p
299	pre-miR-520b
300	pre-miR-520c
301	pre-miR-520d
302	pre-miR-520d*
303	pre-miR-520e
304	pre-miR-520f
305	pre-miR-520g
306	pre-miR-520h
307	pre-miR-521
308	pre-miR-522
309	pre-miR-523
310	pre-miR-524-3p
311	pre-miR-524-5p
312	pre-miR-525-5p
313	pre-miR-525*
314	pre-miR-526a
315	pre-miR-526b
316	pre-miR-526b*
317	pre-miR-532/532-5p
318	pre-miR-539
319	pre-miR-542-3p
320	pre-miR-542-5p

321	pre-miR-544
322	pre-miR-545
323	pre-miR-548a-3p
324	pre-miR-548b-3p
325	pre-miR-548c-3p
326	pre-miR-548d-3p
327	pre-miR-549
328	pre-miR-550*
329	pre-miR-551a
330	pre-miR-551b
331	pre-miR-552
332	pre-miR-553
333	pre-miR-554
334	pre-miR-555
335	pre-miR-556
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342	pre-miR-563
343	pre-miR-564
344	pre-miR-565
345	pre-miR-566
346	pre-miR-567
347	pre-miR-568
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362	pre-miR-583
363	pre-miR-584
364	pre-miR-585
365	pre-miR-586
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368	pre-miR-589*
369	pre-miR-590-5p
370	pre-miR-591
371	pre-miR-592
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389	pre-miR-611
390	pre-miR-612
391	pre-miR-613
392	pre-miR-614
393	pre-miR-615
394	pre-miR-616*
395	pre-miR-617
396	pre-miR-618
397	pre-miR-619
398	pre-miR-620
399	pre-miR-621
400	pre-miR-622

401	pre-miR-623
402	pre-miR-624*
403	pre-miR-625
404	pre-miR-626
405	pre-miR-627
406	pre-miR-628
407	pre-miR-629*
408	pre-miR-630
409	pre-miR-631
410	pre-miR-632
411	pre-miR-633
412	pre-miR-634
413	pre-miR-635
414	pre-miR-636
415	pre-miR-637
416	pre-miR-638
417	pre-miR-639
418	pre-miR-640
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430	pre-miR-652
431	pre-miR-653
432	pre-miR-654-5p
433	pre-miR-655
434	pre-miR-656
435	pre-miR-657
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437	pre-miR-659
438	pre-miR-4983/660
439	pre-miR-661
440	pre-miR-662
441	pre-miR-663
442	pre-miR-668
443	pre-miR-671
444	pre-miR-675
445	pre-miR-7
446	pre-miR-758
447	pre-miR-765
448	pre-miR-766
449	pre-miR-767-3p
450	pre-miR-767-5p
451	pre-miR-768-3p
452	pre-miR-768-5p
453	pre-miR-769-3p
454	pre-miR-769-5p
455	pre-miR-770-5p
456	pre-miR-801
457	pre-miR-802
458	pre-miR-9
459	pre-miR-9*
460	pre-miR-92a
461	pre-miR-92b
462	pre-miR-93
463	pre-miR-95
464	pre-miR-96
465	pre-miR-98
466	pre-miR-99a
467	pre-miR-99b
468	pre-miR-297

Supplementary Table 2

No.	Pre-miRNA
1	**pre-miR-153**
2	**pre-miR-185**
3	**pre-miR-186**
4	**pre-miR-187**
5	**pre-miR-219**
6	**pre-miR-22**
7	**pre-miR-220**
8	**pre-miR-329**
9	**pre-miR-33**
10	**pre-miR-433**
11	**pre-miR-450**
12	**pre-miR-451**
13	**pre-miR-485-3p**
14	**pre-miR-485-5p**
15	**pre-miR-486/486-5p**
16	**pre-miR-487a**
17	**pre-miR-497**
18	**pre-miR-548b-3p**
19	**pre-miR-574**
20	**pre-miR-584**
21	**pre-miR-585**
22	**pre-miR-586**
23	**pre-miR-587**
24	**pre-miR-588**
25	**pre-miR-589***
26	**pre-miR-599**
27	**pre-miR-606**
28	**pre-miR-618**
29	**pre-miR-623**
30	**pre-miR-624***
31	**pre-miR-625**
32	**pre-miR-626**
33	**pre-miR-630**

Supplemetary Table 3

pre-miR oligonucleotides used for validation experiments	**miRBAse Accession**	**Assay ID**
pre-miR-17-5p	MIMAT0000070	MC12412
pre-miR-21	MIMAT0000076	MC10206
pre-miR-23a	MIMAT0000078	AM10644
pre-miR-130b	MIMAT0000691	MC10777
pre-miR-133a	MiMAT0000427	AM10413
pre-miR135a	MIMAT0000428	MH12315
pre-miR-150	MiMAT0000451	MH10070
pre-miR-181a	MiMAT0000256	MH10421
pre-miR-181a*		
pre-miR-181b	MiMAT0000257	PM12442
pre-miR-195	MIMAT0000461	MH10827
pre-miR-200b	MIMAT0000318	AM10492
pre-miR-200c	MiMAT0000617	AM11714
pre-miR-222a	MIMAT0000279	MC11376
pre-miR-602	MIMAT0003270	PM11398
pre-miR-let-7	MIMAT0000062	MC10050
anti-miR-co		
anti-miR-let-7		MH10050
anti-miR-23a		MH10644
pre-miR-Control		
anti-miR-Control		
si-CXCL12		

Primers for cloning of the 3`UTR of CXCL12	
SDF1a_3´UTR_s (SpeI restriction site)	GAA CTA GTG CAC AAC AGC CAA AAA GGA C
SDF1a_3´UTR_as (HindIII ")	CCA AGC TTT AAG CTC CAT CAC TAA CAA C
Primer mRNA targets	
CXCL12	Hs00171022_m1 (life technologies)

Primer miRNA target	
hsa-miR-23a	(000399; Applied Biosystems)
hsa-miR-130b	-456
hsa-miR-135a	-460
hsa-miR-200b	-2251
hsa-miR-200c	-505
hsa-miR-216	-519
hsa-miR-222	-525
hsa-miR-602	-2925

Supplementary Figure legends

Figure S1. Heatmap showing the expression of miR-23a, miR-130b and miR-222 in hBMSCs in comparison to their expression in HSPCs (from BM or CB) and a variety of tissues. Values are normalized to SCP-1 cell line. U6 snRNA served as the internal control.

Figure S2. Schematic representation of the workflow for the selection of candidate CXCL12-regulating miRNAs

Figure S3. TGFB1-associated CXCL12 expression is mediated by the SMAD pathway. Primary hBMSCs were either left untreated or treated with TGFB1 in the presence or absence of SB (SB431542) for 24 h. Cell lysates were analyzed by western blot using an anti-phosphoSmad3 and reprobed with anti-Smad2/3 antibodies. Results shown are representative of 3 independent experiments.

Figure S1

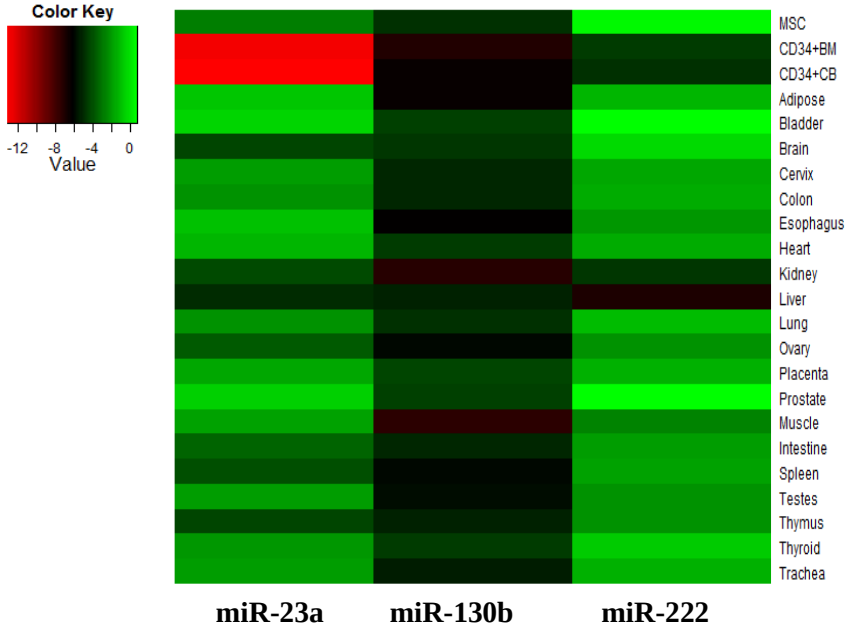
Color Key
-12
-8
-4
0
Value
MSC
CD34+BM
CD34+CB
Adipose
Bladder
Brain
Cervix
Colon
Esophagus
Heart
Kidney
Liver
Lung
Ovary
Placenta
Prostate
Muscle
Intestine
Spleen
Testes
Thymus
Thyroid
Trachea
miR-23a
miR-130b
miR-222

Figure S2

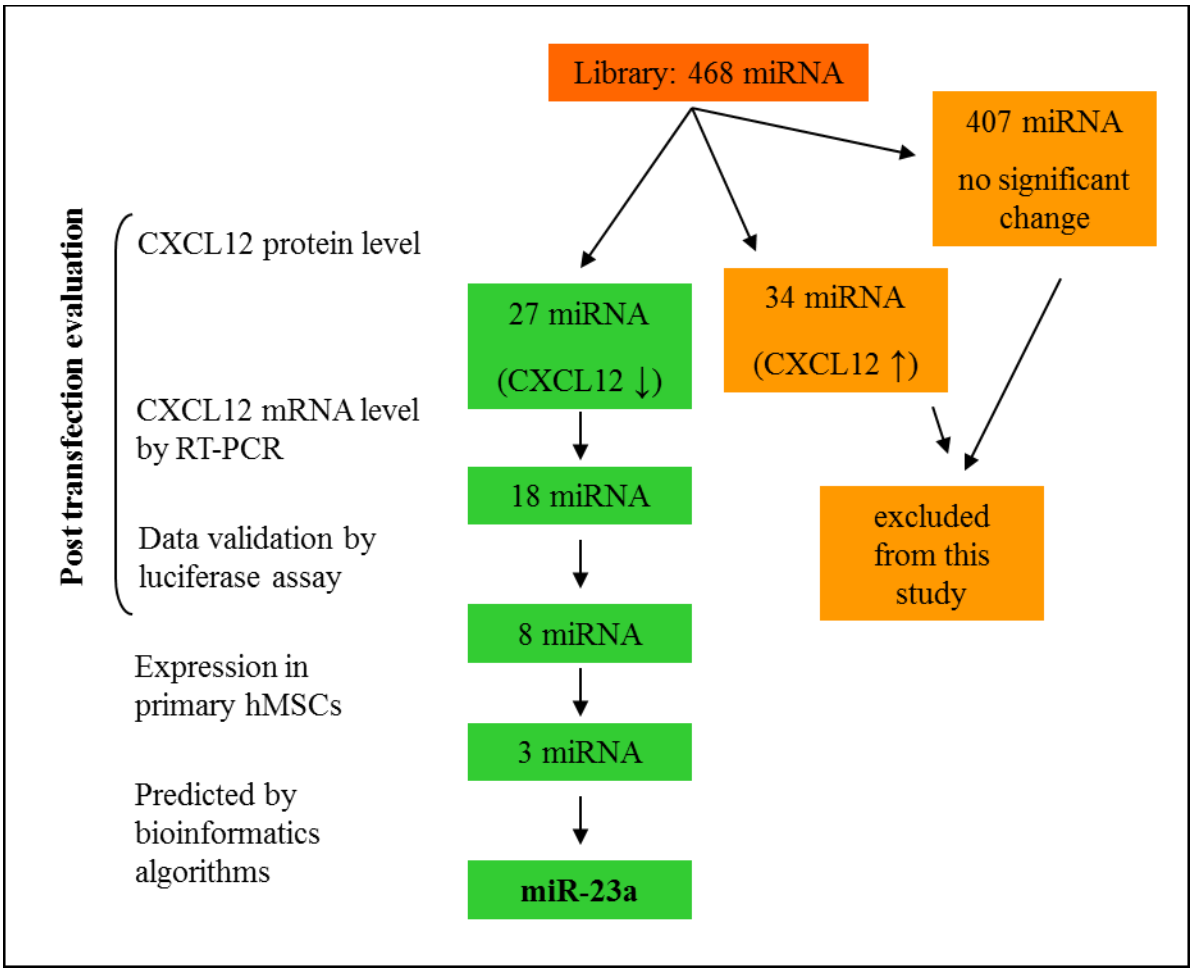
Library: 468 miRNA
407 miRNA
no significant change
34 miRNA
(CXCL12 ↑)
27 miRNA
(CXCL12 ↓)
excluded from this study
Post transfection evaluation
CXCL12 protein level
CXCL12 mRNA level by RT-PCR
Data validation by luciferase assay
Expression in primary hMSCs
Predicted by bioinformatics algorithms
18 miRNA
8 miRNA
3 miRNA
miR-23a

Figure S3