Supporting Information

High-throughput stem cell-based phenotypic screening through microniches

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Supplementary text

Assessment of microniche functionality using a fluorescent reporter system

Transiently engineered HEK-IL4-YFP reporter cells were cultured at different concentration of soluble IL4-Fc. After 48 hours, cell nuclei were stained with Hoechst and samples were imaged in the DAPI and GFP channel (Figure S1a). Automated quantification of the YFP signal normalized to the cell number via Hoechst staining revealed a significant increase in YFP expression and thus successful IL4-Fc signaling at concentrations as low as 10 ng/ml (Figure S1b). Automated detection and thresholding of the fluorescent signal normalized to the cell number via Hoechst staining enabling the quantification of fluorescence is shown (Figure S1c).

Genotypic analysis via multiplexed RT-PCR

By multiplexing several primer pairs, mRNAs encoding for different growth factors can be amplified in one reaction. Fc-tag-encoding mRNA can be amplified with a universal Fc-specific primer followed by amplification cycles with growth factor-specific primers. A second amplification round with nested primers in separated reactions avoids competition between amplicons (Figure S3). To validate the process, a stable cell line recombinant for IL4-Fc was induced, lysed and multiplexed for mRNA encoding for β-actin (positive control) and IL4-Fc. PCR product of the expected size could be detected for both constructs by gel electrophoresis (Figure S3b). Next, microniches recombinant for IL4-Fc were cultured, induced with doxycycline and one microniche was manually picked. In parallel, 500 and 25 control cells recombinant for IL4-Fc were collected, and Fc-encoded gene expression was successfully retraced by multiplex RT-PCR in all samples (Figure S3c).

Recapitulation of primitive streak formation in mouse embryonic stem cells

During gastrulation, mesendodermal progenitors originate in the primitive streak, a transient embryonic structure on the posterior site of the epiblast (Figure S2a). First, we evaluated adhesion of mESC reporter line expressing Brachyury-mCherry (Bra-mCherry), a marker of the primitive streak cultured on hydrogel substrates modified with different ECM components.[1] Hydrogel matrices functionalized with Laminin511 in combination with FN9-10 fragment promoted good adhesion when mESCs were cultured for five days in chemically defined N2B27 medium (data not shown). Previous studies have revealed the role of BMP4, ActA, TGFβ, Wnt3a and canonical Wnt signalling agonist CHIR99021 (CHIR) as potent inducers of primitive streak formation.[2] Hence, mESC adhesion and differentiation on the microgel substrates was tested by addition of the selected Fc-tagged mesendodermal inducers from our Fc-tagged chimeric protein library. mESCs were seeded onto microgels functionalized with Laminin511 and FN9-10 fragment in a low adhesion 96-well plate. To maintain cell adhesion on microgels, the additional supplement of 1% KnockOut™ Serum Replacement and Y-27632, a ROCK inhibitor, to the basal N2B27 medium (here termed advanced medium) was required. From day two onwards FGF2 in absence or presence of the indicated factors was added to the culture according to published protocols.[3] Medium was changed at day four and cell nuclei were labelled with Hoechst to normalize the emergence of the primitive streak marked by the Bra-mCherry signal to the cell number at day five. Mesendodermal differentiation increased upon additional administration of ActA, Wnt3a compared to a negative control condition with FGF2 addition alone (Figure S2b and c). Medium compatibility of cell-loaded microniches in mESC medium was assessed in a preliminary study via live dead staining. Overall, these proof-of-concept experiments demonstrate that the viability seems to be comparable with the viability under normal medium conditions.

Supplementary Figures

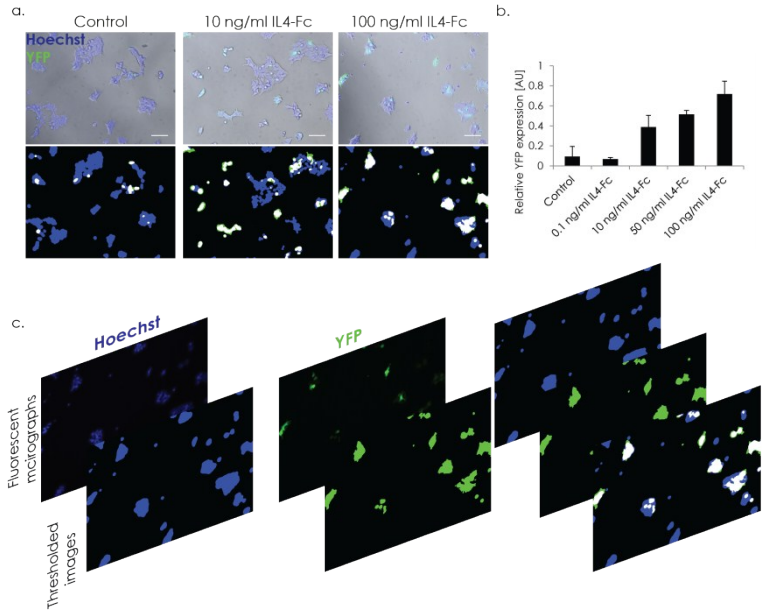

Figure S1 Quantification of the phenotypic effects. a) HEK-IL4-YFP cells were cultured in tissue treated 96 well plates and exposed to different IL4-Fc concentrations. After two days, samples were stained with Hoechst and imaged in the GFP and DAPI channel. b) Automated image-based quantification showed increased YFP signals at a IL4-Fc concentration starting from 10 ng/ml in a dose-dependent manner. c) Fluorescent visualization of HEK-YFP reporter cell nuclei via Hoechst staining in the DAPI channel and YFP signal in the GFP channel with a Axio Observer microscope (Zeiss). Images are automatically thresholded using the triangle filter and quantified in a customized ImageJ plugin. Thresholded images are shown with blue and green masks. The white mask shows overlapping regions of both signals. The same quantification procedure was applied on images with an inverted confocal microscope (Zeiss, 700 LSM) of stained mESC nuclei and Bra-mCherry signal to visualize patterns and 3D-labeled microgels.

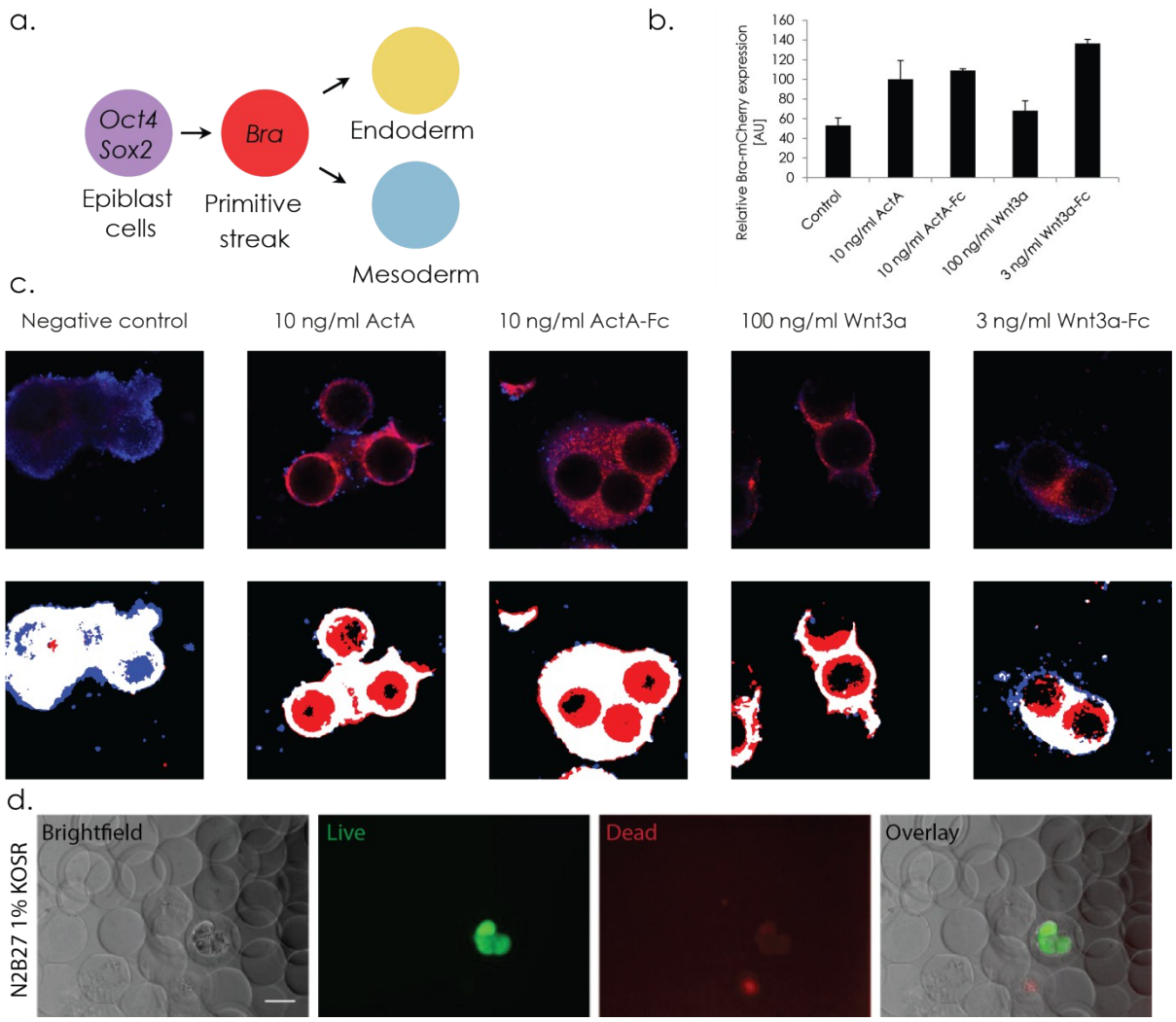

Figure S2 mESC adhesion and differentiation with bioactive ActA-Fc and Wnt3a-Fc on microgels. a) During gastrulation, the primitive streak emerges from the epiblast and gives rise to mesendodermal progenitors with Brachyury as a marker of mesendodermal commitment. b) mESCs were seeded on microgels functionalized with 0.05 mg/ml Lam511 and 0.15 mg/ml FN9-10 at a density of 4000 cells/cm^2 in advanced medium. After two days, FGF2 and indicated morphogens were added. At day four, medium was changed and at five day in culture, cell nuclei were stained with Hoechst dye and imaged in the red and DAPI channel for quantification. Signal intensity of Bra-mCherry was normalized to the cell number by Hoechst staining and analyzed at day five. Normalization of Bra-mCherry to the cell number by the nuclei stain and quantification revealed upregulation after five days in culture compared to a negative control for all the ActA and Wtn3a commercially available as well as from our Fc-fusion protein library. d) Cell aggregate-loaded microniches were transferred 1 day after co-encapsulation into basal mESC medium and a live dead stain (Calcein-green and PI staining) shows partial survival after five days comparable to studies in standard culture medium.

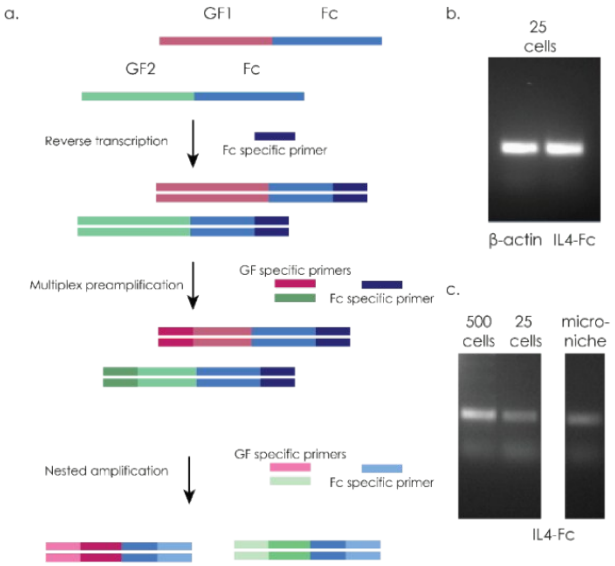

Figure S3 Genotypic analysis via multiplexed PCR. a) In a simultaneous reaction, reverse transcription with a universal Fc-specific primer is followed by a multiplexed preamplification. The reaction is diluted and distributed to separated reaction mixtures for a nested amplification of different transcripts. b) Genotypic identity is assessed after 24 hour doxycycline induction periods for 25 cells by multiplexed PCR for β-actin and IL4-Fc, as well as for c) 500 and 25 cells as well as microniches after a 6-day culture period, shown by electrophoresis in a 1.5% agarose gel.

Experimental Section

Table S1 Plasmid library encoding already generated Fc-tagged fusion proteins provided by the Wilfried Weber lab and Wnt3a, mFGF2 and hInhA from EPFL laboratories.

Plasmid	Protein	Size (bp)
pLMK135	SEAP-Fc (Reporter gene)	2310
pLMK134	PDGFBB –Fc	1362
pLMK133	NCAD –Fc	2964
pLMK014	DKK1-Fc	1590
pEC020	IL4-Fc	1239
pLMK040	IGFI-Fc	1251
pLMK056	Wnt10b –Fc	1365
pLMK151	IL1α-Fc	1332
pLMK022	TGFβ1-Fc	1962
pLMK024	BMP2-Fc	1980
pLMK036	BMP4-Fc	2016
pLMK048	Myostatin-Fc	1917
pLMK020	IGFII-Fc	1332
pLMK124	TSH -Fc	1206
pLMK131	DLL4-Fc	2379
pLMK142	SHH(N-terminal) –Fc	1383
p6266	mWnt3a	1056
p4575	mFGF2	465
pENTR221.INHBA	hInhA	1278

Table S2 Primer for cloning strategy into the pcDNAFRT/TO vector.

primer	sequence
oLK1	CTTGGTACCGAGCTCGGGATCATCCCTTCACCATG
oLK2	CCCTCTAGACTCGAGCCCATAATAGCTGTTTGCCATC
oLK5	GCTTGGTACCGAGCTCGATGGGTCTCACCTCCCAAC
oLK6	CCCTCTAGACTCGAGCATGGTGATGGTGATGATGCG
oLK44	CTTGGTACCGAGCTCG CGCGCTCAAGCCGGCGAT
oLK45	GGACCGTTATAGTTACC CTTGCAGGTGTGCACGTCATAG
oLK46	GTGCACACCTGCAAG GGTAACTATAACGGTCCTAAGGTAG
oLK47	CTTGGTACCGAGCTCG CCGCCGCGGAAGGGCCAT
oLK48	GGACCGTTATAGTTACC GCTCTTAGCAGACATTGGAAG
oLK49	CAATGTCTGCTAAGAGC GGTAACTATAACGGTCCTAAGGTAG
oLK50	CTTGGTACCGAGCTCG CCACCATGCCCTTGCTTTG
oLK51	GGACCGTTATAGTTACC TGAGCACCCACACTCCTCC
oLK52	GAGTGTGGGTGCTCA GGTAACTATAACGGTCCTAAGGTAG

Table S3 Plasmid library encoding Fc-tagged fusion proteins cloned into the FlpIn expression vector pcDNA5/FRT/TO to generate Flp-In™ T-REx™ transgenic cell lines.

Plasmid	Protein	Size (bp)
pLK5	SEAP-Fc	2310
pLK6	PDGF-BB-Fc	1362
pLK7	N-CAD-Fc	2964
pLK8	DKK-Fc	1590
pLK9	IL4-Fc	1239
pLK10	IGF1-Fc	1251
pLK11	Wnt10b-Fc	1365
pLK12	IL1a-Fc	1332
pLK13	Tgfb-Fc	1962
pLK14	BMP2-Fc	1980
pLK15	BMP4-Fc	2016
pLK17	Myostatin-Fc	1917
pLK18	IGF2-Fc	1332
pLK19	TSH-Fc	1206
pLK20	DLL4-Fc	2379
pLK21	SHH-Fc	1383
pLK23	mWnt3a-Fc	1827
pLK24	mFGF2-Fc	1254
pLK25	hInhA-Fc	2070

Table S4 Multiplex PCR primers. Outer primers are for RT and preamplification and inner primers for nested amplification.

primer	sequence	description
oLK13	GTCTTCCCCTCCATCGTG	forward primer for bactin for outer amplicon
oLK14	CTCGTCATACTCCTGCTTGC	reverse primer for bactin for outer amplicon
oLK15	GCGAGAAGATGACCCAGAT	forward primer for bactin for inner amplicon
oLK16	GTCCATCACGATGCCAGT	reverse primer for bactin for inner amplicon
oLK17	CTTCCCCCTCTGTTCTTCC	forward primer for outer amplicon for IL4-Fc
oLK18	AGTCGTGTGGTGATTGTGC	reverse primer for outer amplicon for all Fc-constructs
oLK19	CCGAGTTGACCGTAACAGAC	forward primer for inner amplicon for IL4-Fc
oLK20	CCTTCTCATGGTGGCTGTAG	reverse primer for inner amplicon for IL4-Fc
oLK21	CGCTCCTTTGATGATCTCC	forward primer for outer amplicon for PDGF-BB-Fc
oLK22	GAGATTGTGCGGAAGAAGC	forward primer for inner amplicon for PDGF-BB-Fc
oLK23	CACCACTCGCTACCTTAGGA	reverse primer for inner amplicon for all Fc-constructs
oLK24	GGGCACTGACTGAGACGAT	forward primer for outer amplicon for SEAP-Fc
oLK25	CAGGAGCAGACCTTCATAGC	forward primer for inner amplicon for SEAP-Fc
oLK26	CTCGGAGATGGTGGTAGAGA	forward primer for outer amplicon for Wnt3aFc
oLK27	GAAATGCCACTGTGTTTTCC	forward primer for inner amplicon Wnt3aFc
oLK28	CTTGGAGTGTGATGGCAAG	forward primer for outer amplicon ActA/InhAFc
oLK29	GAGACCCATGTCCATGTTG	forward primer for inner amplicon ActA/InhAFc
oLK30	TCCTCCTCGCATCTCTTCTA	forward primer for outer amplicon IGF1-Fc
oLK31	ATGCCCAAGACCCAGAAG	forward primer for inner amplicon IGF-1-Fc
oLK32	GTGTGCTTGACGCTGTCC	forward primer for outer amplicon Wnt10bFc
oLK33	CAGTGGTGAGCAGGATCG	forward primer for inner amplicon Wnt10bFc
oLK34	TGACGCCCTCAATCAAAGTA	forward primer for outer amplicon IL1alpha
oLK35	AGCAAGACTACTGGGTGTGC	forward primer for inner amplicon IL1alphaFc
oLK36	CCTTTCCTGCTTCTCATGG	forward primer for outer amplicon TGFbetaFc
oLK37	GCCCTGTACAACCAGCATAA	forward primer for inner amplicon TGFbetaFc
oLK38	TGGAGGAGAAACAAGGTGTC	forward primer for outer ampliconBMP2Fc
oLK39	GATGCTGTACCTTGACGAGAA	forward primer for inner amplicon BMP2Fc
oLK40	CTCAAGGGAGTGGGAATTG	forward primer for outer ampliconBMP4Fc
oLK41	CTGAACTGAGTGCCATCTCC	forward primer for inner amplicon BMP4Fc

Table S5 Barcoded mRNA seq primers for RT and preamplification with template switch oligo (E3V6NEXT) and respective barcoded E3V6XX primer specific for the Fc-tag encoding transcriptome, cDNA amplification SINGV6 annealing at both ends and P5NEXTPT5 to replace the i5 primer during sequencing library preparation.

primer	sequence
E3V6A1	5Biosg/ACACTCTTTCCCTACACGACGCTCTTCCGATCT AAAACT GAGGACAGGGCTTGATTGT
E3V6NBA3	ACACTCTTTCCCTACACGACGCTCTTCCGATCT AAAGTT GAGGACAGGGCTTGATTGT
E3V6NEXT	5'-iCiGiCACACTCTTTCCCTACACGACGCrGrGrG-3'
E3V6A5	ACACTCTTTCCCTACACGACGCTCTTCCGATCT AAATTG CATTTGCATGGAGGACAGGGCTTG
E3V6A6	ACACTCTTTCCCTACACGACGCTCTTCCGATCT AACAAT CATTTGCATGGAGGACAGGGCTTGATTGT
E3V6A7	ACACTCTTTCCCTACACGACGCTCTTCCGATCT AAGATT CATTTGCATGGAGGACAGGGCTTGATTGT

E3V6A8	ACACTCTTTCCCTACACGACGCTCTTCCGATCT AAGTAT CATTTGCATGGAGGACAGGGCTTGATTGT
E3V6A9	ACACTCTTTCCCTACACGACGCTCTTCCGATCT AATACA CATTTGCATGGAGGACAGGGCTTGATTGT
E3V6A10	ACACTCTTTCCCTACACGACGCTCTTCCGATCT AATAGT CATTTGCATGGAGGACAGGGCTTGATTGT
SINGV6	5'-/5Biosg/ACACTCTTTCCCTACACGACGC-3'
P5NEXTPT5	5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCG*A*T*C*T*-3'

References

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[3] aD. A. Turner, P. C. Hayward, P. Baillie-Johnson, P. Rue, R. Broome, F. Faunes, A. Martinez Arias, *Development* **2014**, *141*, 4243-4253; bD. A. Turner, J. Trott, P. Hayward, P. Rué, A. Martinez Arias, *Biology Open* **2014**.