Gene expression profiling of differentiating embryonic stem cells expressing dominant negative fibroblast growth factor receptor 2

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Abstract

Embryonic stem (ES) cells are derived from the inner cell mass of the blastocyst and can be cultured as three-dimensional embryoid bodies (EBs) in which embryonic pregastrulation stages are faithfully mimicked. Fibroblast growth factor receptors (mainly FGFR2) are involved in the first differentiation events during early mammalian embryogenesis. It has been demonstrated that the presence of FGFR2 is a prerequisite for laminin-111 and collagen type IV synthesis and subsequently basement membrane formation in EBs. To identify genes that are influenced by FGFR signalling, we performed global gene expression profiling of differentiating EBs expressing dominant negative FGFR2 (dnFGFR2), acquiring an extensive catalogue of down- and up-regulated genes. We show a strong down-regulation of endodermal and basement membrane related genes, which strengthen the view that the FGFR signalling pathway is a main stimulator of basement membrane synthesis in EBs. We further present down-regulation of genes previously not linked to FGFR signalling, and in addition an active transcription of some mesodermal related genes in differentiating dnFGFR2 EBs.

Keywords: FGFR signalling; microarray; gene expression; basement membrane; ES cell differentiation; embryoid bodies

1. Introduction

The transformation of inner cell mass into columnar epithelium can be studied in vitro by the well-established system of embryoid body (EB) culture (Desbaillets et al., 2000). The spontaneous differentiation of embryonic stem (ES) cells into EBs mimics the morphological changes occurring in early embryonic development. ES cell derived EBs are similar to the egg-cylinder embryo, and upon aggregation they differentiate into round structures consisting of the three embryonic germ layers**,** namely the endoderm, ectoderm and the mesoderm. The endoderm synthesises the components of the basement membrane (BM), which separates the endoderm from the ectoderm (Coucouvanis and Martin, 1995).

Fibroblast growth factor receptors (FGFR) belong to a family of four membrane bound receptor tyrosine kinases (FGFR1-4) that mediate signals of at least 22 fibroblast growth factors (FGF) (Eswarakumar et al., 2005). FGFs/FGFRs play important roles in multiple biological processes including early embryonic development, cell growth and migration, organ formation and bone growth (Deng et al., 1994; Feldman et al., 1995; Arman et al., 1998; Sun et al 1999; Powers et al., 2000; Lonai, 2005; Thisse and Thisse, 2005).

Previous work demonstrated an essential role for FGFR signalling during EB development (Chen et al., 2000; Li et al., 2001a; Li et al., 2001b; Li et al., 2004). It was reported that ES cells expressing dominant negative FGFR2 (dnFGFR2) fail to develop the two characteristic cell layers of the EB, the endoderm and the ectoderm (Chen et al., 2000). Moreover, normal FGFR signalling was shown to be important for the genetic regulation of the BM proteins laminin-111 and collagen type IV, and consequently, BM formation (Li et al., 2001a). A decisive intermediate event for laminin-111 synthesis is activation of Akt/PKB, a cell survival kinase. Akt acts

presumably upstream of the GATA4/6 transcription factors, which induce synthesis of the three genes (Lama1, Lamb1-1 and Lamc1) encoding laminin-111 (Li et al., 2001b; Li et al., 2004). In turn, laminin-111 induces development of the ectoderm (Li et al., 2001a; Li et al., 2002; Miner et al., 2004; Scheele et al., 2005; Alpy et al., 2005). Endoderm development is thus the target of FGF action, where the signalling pathway leading to laminin-111 production is essential for ectoderm development.

In this study we attempted to identify genes that are influenced by FGFR signalling in developing EBs in vitro. We performed an Affymetrix GeneChip (MOE430 2.0) microarray analysis during four days of spontaneous differentiation of wild type ES cells and ES cells expressing dnFGFR2 (Chen et al., 2000). The four-day time period of ES cell differentiation corresponds to the developmental events occurring between implantation and gastrulation of mouse embryos. We found that mainly the biosynthetic machinery for endoderm and BM development was abrogated in dnFGFR2 EBs. We further detected active gene transcription in dnFGFR2 EBs that were related to mesodermal inducers. In addition, we present several down- and up-regulated genes previously not associated with FGFR signalling.

2. Results and Discussion

2.1. Hierarchical clustering of developing wild type and dnFGFR2 EBs

The R11 ES cell line (wild type) and its derivate, the 1c6 ES cell line (dnFGFR2) were allowed to differentiate spontaneously into EB during four days of culture. One set of cultured ES cells were divided into four bacterial culture dishes in LIF depleted cell culture medium on day 0, which marks the beginning of the ES cell differentiation events (Fig. 1). Cell culture was repeated three times for the two ES cell lines. RNA was isolated two hours after the initial seeding of ES cells to bacterial culture plates at day 0, then 24 (day 1), 48 (day 2) and 96 hours (day 4) from each replicate, and processed for microarray analysis.

The microarray raw data was analysed using the dChip software. After normalization and calculation of expression values the samples were clustered using the hierarchal clustering method, which provided a quality control for the three independent biological replicates. Before the sample clustering, a filter was applied to exclude probe sets with low expression level and low variation across samples. Out of 45101 probe sets, 3938 remained after filtration. The clustering dendogram (Fig. 2) revealed that R11 day 0 samples and 1c6 day 0 to day 4 samples comprised a distinct subgroup, whereas R11 day 1 to day 4 samples were subgrouped together. As it has previously been shown that 1c6 EBs fail to form endoderm and ectoderm (Chen et al., 2000), the hierarchical clustering correctly clustered the replicates by their appropriate developmental stages.

2.2. Identification of differentially expressed genes and EASE analysis

In order to investigate the gene expression differences between R11 and 1c6 cell lines, we conducted a pair-wise comparison of these two conditions at each time point.

From the 45101 probe sets, a gene was considered differentially expressed if it met the criteria described in materials and methods. The number of genes differentially expressed between the two conditions increased over the four days of EB development. Genes positively regulated by FGF-signalling, and thus down-regulated in 1c6 as compared to R11 EBs, were more abundant than the up-regulated genes. The number of down-regulated genes was 183, 252, 513, and 887 at day 0, day 1, day 2 and day 4, respectively (Tables S1, S3, S5 and S7). Genes up-regulated in 1c6 as compared to R11 were 62, 43, 90, and 212 at day 0, day 1, day 2 and day 4, respectively (Tables S2, S4, S6 and S8).

To better understand the developmental processes throughout the four-day time period of EB development, each of the gene lists were analysed with the EASE tool. EASE accepts a list of differentially expressed genes, without any consideration to fold change, and performs biological "theme" finding using gene category over-representation analysis. We chose to set the cut-off for the EASE score to 0.05, thus obtaining only the most significant categories for biological processes, cellular components and molecular functions (Tables S9-16). When analysing the down-regulated genes in the 1c6 EBs, the most significant biological processes from day 0 to day 4 were development, imprinting, one-carbon compound metabolism, and lipid transport, respectively. At all time points, the most significant category for cellular component was BM. Among the up-regulated genes, the most significant biological processes from day 0 to day 4 were development, cholesterol metabolism, and transcription, respectively.

2.3. Influence of the FGFR2 mutation on gene expression studied by time series analysis

From the lists of down-regulated genes in the 1c6 cell line (Tables S1, S3, S5 and S7), we present the top 20 genes with the highest significant negative foldchanges.

When examining the down-regulated genes, the results showed striking emergence of endodermal and BM regulatory genes as early as day 0, when the compaction and differentiation of ES cell begins (Table 1). The genes for Sox17, Foxa2, Lama1, Lamb1-1, Gata6, Col4a1 and Col4a2 have all been shown to be important during endoderm and BM development (Niimi et al., 2004; Futaki et al., 2004; Sinner et al., 2004; Li et al., 2001a; Li et al., 2004; Poschl et al., 2004). Most of these genes are directly involved in the initial steps of embryonic development (Miner et al., 2004; Scheele et al., 2005; Zhao et al., 2005) and in addition, they have also been shown to be affected by FGFR signalling (Li et al., 2001a; Li et al., 2004). Thus, our data strengthen the view that the FGFR signalling pathway is a main stimulator during the synthesis of BM in developing EBs. Another notable down-regulated gene associated with FGF signalling was Flrt3, which encodes a leucine rich repeat transmembrane protein that has been shown to form complex with FGFR, thereby promoting FGF signalling during Xenopus development as well as during mouse development (Böttcher et al., 2004; Haines et al., 2006). It is thus tempting to speculate that Flrt3 plays a crucial role during early embryonic development, when the embryonic germ layers are forming. We also detected down-regulation of Gja1, which is regulated by FGFs in astroglial junctions (Reuss et al., 2000). Furthermore, we observed a number of genes that have not been coupled to FGFR signalling so far, but could have important roles during the implantation period in mouse embryogenesis. The expression of genes Prg1, Foxq1, Spink3, Glipr1, Ctsc, Mael,

Car2 and Efhc2 was significantly lower in day 0 1c6 EBs compared to R11 EBs. Yet, we cannot exclude that the observed down-regulation of these genes may be due secondary effects of defect FGFR signalling.

Some of the genes down-regulated at day 0 (Prg1, Sox17, Foxq1, Gata6, Col4a1, Lamb1-1, Foxa2*)* remained down-regulated at day 1 of EB development, the time point when the endoderm begins to form. Also, new genes entered the top 20 down-regulated gene list (Table 2). We detected Dab2 that presented the highest negative foldchange at day 1. Dab2 is a mitogen responsive phosphoprotein that has a role in visceral endoderm cell organisation during embryogenesis (Yang et al., 2002), and it was shown to be a downstream target for Gata6 (Morrisey et al., 2000). The expression of Sox7, which plays a key role in the regulation of the transcription factors Gata4 and Gata6 (Futaki et al., 2004), was down-regulated in 1c6 EBs. Also, Pdgfra, whose expression is induced by Gata4 (Wang and Song, 1996), was down-regulated at day 1 of 1c6 EB development. We also noted down-regulation of Grb10 that has been shown to play a role in Akt activation (Jahn et al., 2002). Chen et al (2000) demonstrated that FGFR sends signals through the Akt rather than the Mapk/Erk pathway in developing EBs, and later it was shown that constitutively active Akt triggered massive secretion of laminin-111 and collagen type IV in EBs (Li et al., 2001b). Thus, Grb10 might possibly be involved in FGFR signalling and the activation of the laminin-111 and collagen type IV encoding genes. The expression of Tspan8, which might be a FGF10 target in embryonic lung epithelium (Lü et al., 2005), was also decreased in 1c6 EBs. Whether the decreased expression of the genes Nostrin (LOC329416), Bmper and Pthr1 is a direct effect of deficient FGFR signalling remains to be elucidated. However, they have been linked to developmental processes during embryogenesis (Choi et al., 2005; Moser et al., 2003; MacLean and

Kronenberg, 2005). Also, the expression of Clic6 and Tcfec was down-regulated at day 1 of 1c6 EB development.

At day 2, when the endoderm is already formed and the BM components start to be secreted and assembled, Sox17, Tcfec, Sox7, Lama1, Flrt3, Clic6, Dab2, Nostrin, Spink3, Grb10 and Prg1 remained down-regulated (Table 3). In addition, we detected highly significant down-regulation of Dkk1 and Amn. These genes have been shown to play important roles during early embryonic development (Mukhopadhyay et al., 2001; Kalantry et al., 2001). Interestingly, in transgenic mice displaying mutant Fgfr1 gene, the expression of Dkk1, which is a Wnt signalling antagonist, was down-regulated (Hajihosseini et al., 2004), much like in our 1c6 EBs. Ttr and Cubn that are expressed during endoderm development (Abe et al., 1996; Drake et al., 2004) and Amot, that acts during morphogenetic movements of visceral endoderm in mouse embryos (Shimono and Behringer, 2003), were also highly down-regulated in 1c6 EBs at day 2. We also observed down-regulation of Ctsh, Pga5 and Car4 that might indicate a lack of morphogenetic remodelling in the extracellular matrix of the 1c6 EBs.

At day 4, when the BM assembly is complete and the polarization of the ectoderm is ongoing, Sox17, Lama1, Gata6, Flrt3, Pdgfra, Sox7, Pga5, Grb10, Cubn, Foxa2, Ctsh and Foxq1 continued to display down-regulated gene expression patterns (Table 4). In addition, new genes entered the top 20 list for down-regulated genes. The expression of S100g, Aqp8, Afp, Enpep, Klb, Cited1 and Lgals2 was low in 1c6 EBs at day 4 compared to R11 EBs. Cited1 is a transcriptional modulator expressed in the anterior visceral endoderm and mesoderm during embryonic development (Dunwoodie et al., 1998) and it was highly expressed during BM development in Engelbreth-Holm-Swarm tumours and F9 embryonal carcinoma cells (Futaki et al.,

2003). Expression of Afp, which is an endoderm marker, was absent in Foxa2 mutant mouse embryos (Lee et al., 2005). Also, we noted down-regulation of Lgals2, which is a carbohydrate binding protein that may bind to cell adhesion molecules (Hughes, 2001) and Aqp8 that has a role in fluid homeostasis during mouse embryo implantation (Richard et al., 2003).

Form day 0 to day 4, we also found the uncharacterised genes 1110032E23Rik, 4933402E13Rik and 1200002N14Rik with decreased expression patterns.

2.4. Mesodermal gene activity in FGFR mutant 1c6 EBs

The microarray data also provided details of active gene expression patterns in 1c6 EBs. From the lists of up-regulated genes in 1c6 EBs form day 0 to day 4 (Tables S2, S4, S6 and S8), we chose to present the top 10 genes with significant positive foldchange patterns (Tables 1-4).

Interestingly, 1c6 ES cells are able to aggregate into EBs and survive for several days during cultivation, before they disintegrate spontaneously. Although 1c6 EBs fails to form endodermal and ectodermal structures, the outer cells of these EBs were able to recognize external differentiation signals that lead to ectoderm polarization (Li et al., 2001a). Studies pointed toward the fact that 1c6 EBs are composed of undifferentiated homogenous stem cell aggregates (Li et al., 2001a; Li et al., 2004).

Indeed, our microarray data confirmed the lack of gene transcription related to endoderm, although we found active transcription of genes related to the developing mesodermal cell lineages.

Frequently reoccurring genes throughout the four days of 1c6 EB development were Mest, Nnat and 4930422N03Rik. Mest (Peg1) is an imprinted mesodermal related gene that was shown to play a role in epithelial-mesenchymal interactions (Kanwar et al., 2002). Nnat (Peg5) is another imprinted gene that is expressed in the developing nervous system (Wijnholds et al., 1995), while 4930422N03Rik is yet to be characterized.

From day 0 to day 1, we observed an up-regulation of Sfrp1, which is a Wnt signalling antagonist and has role in anterior-posterior patterning during mouse embryonic development (Satoh et al., 2006). The expression of Kbtbd11, an uncharacterized gene, was up-regulated from day 0 to day 2. Up-regulation of Odz3, which is expressed in mesodermally derived tissues and developing nervous system (Ben-Zur et al., 2000) was noticed at day 1 and day 2, while the expression of Vegfc that has been suggested to play role in embryo venous development (Kukk et al., 1996) was observed from day 2 to day 4 of 1c6 EB development.

We also observed expression of Trps1 at day 2. During mouse development, Trps1 is expressed in cartilage, hair follicles and brain (Kunath et al., 2002). Expression of Nr5a2, which was detected at day 4 in 1c6 EBs, was shown to be required for activation and maintenance of Oct4 expression in undifferentiated ES cells and ectoderm-derived cells in the developing embryos (Gu et al., 2005).

These results suggest an active transcription of mesodermal genes that usually are up-regulated at later stages of development. We also emphasize the up-regulation of stemness genes that are normally suppressed during the early developmental events (consult the complete lists in Tables S2, S4, S6 and S8).

2.5. Confirmation of gene expression differences by RT-PCR

To verify the gene expression differences observed in the microarray analysis between the R11 and 1c6 ES cell lines, we randomly selected 13 genes from Tables 1 to 4. The expression of Bmper, Dab2, Dkk1, Flrt3, Gata6, Nostrin, Pdgfra, Sox7, 1110032E23Rik, Mest, Nr5a2, Trps1 and Vegfc at day 0 and day 4 was confirmed by semiquantitative RT-PCR (Fig. 3). There was a good correlation between the microarray and the RT-PCR data, indicating a reliable depiction of gene activation and of transcription levels during EB development.

To further validate the effects of defect FGFR signalling, we cultured another dnFGFR2 ES cell clone, called 1c3 (Chen et al., 2000; Li et al., 2001a). The 1c3 and its parental line R11 were subjected to the same differentiation-promoting conditions as the 1c6 ES cells. The RT-PCR analysis showed down-regulation of Bmper, Dkk1, Flrt3, Gata6 and 1110032E23Rik mRNAs in developing 1c3 EBs compared to R11 EBs, while Mest and Nr5a2 mRNAs were up-regulated (Suppl. Fig. 1). Thus, gene expression during 1c3 and 1c6 EB development is strikingly similar. These results indicate that the gene expression differences between the R11 and the dnFGFR2 ES cell clones 1c3 and 1c6 are not due to unspecific changes that may have occurred in the transfected cells prior to the experiments reported, but instead are associated with FGFR signalling defects.

3. Conclusion

In summary, it has previously been shown that a dominant negative mutation in FGFR2 leads to loss of laminin-111 and collagen type IV in developing EBs and as a consequence, BM assembly and ectoderm differentiation is abrogated (Li et al., 2001a). Yet, the global gene expression changes upon introduction of dnFGFR2 into ES cells have remained unknown. Recently, only preliminary data from a less exhaustive microarray experiment was published (Li et al., 2004). Now, we report an extensive catalogue of differentially expressed genes. The strong and early dominance of BM genes as FGFR signalling targets is striking. We expected that FGFR signalling first would activate other types of genes (Thisse and Thisse, 2005). However, of the top 20 down-regulated genes at day 0, about 30% of the genes encode proteins involved in laminin-111 and collagen type IV biosynthesis. The expression pattern of Lama5 was similar in R11 and 1c6 EBs (not shown), which indicates that not all laminins but mainly expression of laminin-111 is dependent on the FGFR signalling pathway. Heparan sulphate proteoglycans (HSPG) are important components of the BM. Furthermore, HSPG facilitate FGFR signalling (Thisse and Thisse, 2005). In accordance with previous findings in 1c3 EBs (Li et al., 2001a), the expression of genes encoding the HSPGs perlecan (Hspg2) and agrin (Agrn) was not altered (not shown), while genes coding for glypicans (Gpc3 and Gpc6) were significantly down-regulated. We also detected down-regulation of enzymes involved in HSPG synthesis such as Hs3st1 and Has2, and genes coding for HSPG interacting proteins such as Habp2 and Sdcbp (Tables S1, S3, S5 and S7). These data further support the notion that FGFR signalling affects BM synthesis and assembly in developing EBs.

Several highly down-regulated genes have previously been associated with FGFR signalling (Flrt3, Gja1, Dkk1, Tspan8) but we also detected many down-regulated genes that have not been coupled to FGFR signalling so far (Grb10, Prg1, Foxq1, Tcfec, Dab2, Amot). Whether these genes are directly affected by defect FGFR signalling during EB development remains to be tested. Finally, several novel genes were strongly influenced by FGFR signalling (1110032E23Rik, 1200002N14Rik, 4933402E13Rik). These genes will be further characterized and their roles in early embryonic development established.

4. Experimental Procedures

4.1. Cell lines and cell cultures

Mouse ES cell clones were a kind gift from Dr. Peter Lonai (Weizmann Institute of Science, Israel). The wild type (R11) and the dnFGFR2 (1c3 and 1c6) ES cell lines were described previously (Chen et al., 2000; Li et al., 2001a).

ES cell clones were cultured as described before (Chen et al., 2000), with some modifications. Briefly, 1 x 10^7 undifferentiated ES cells were seeded on tissue culture plates in ES cell medium for 24 hours to remove the STO fibroblasts. The cell clumps formed were then detached by pipetting and transferred to bacteriological plates in ES cell medium without LIF. The day of transfer of primary aggregates was denominated as day 0. Cell cultures were repeated three times at different time points for the ES cell lines.

4.2. RNA extraction and hybridization to microarray chips

Two hours after transfer (day 0), the first cell population was harvested. Thereafter, EBs were harvested after 24 (day 1), 48 (day 2) and 96 hours (day 4) of culture. Total RNA was extracted with TRIzol® Reagent (Life Technologies) and RNeasy MinElute Cleanup Kit (Qiagen) according to the manufacturer's instructions.

For microarray analysis the R11 and the 1c6 ES cell lines were used. Total RNA was processed for microarray hybridisation and hybridised to Affymetrix MOE430 2.0 mouse GeneChip (Santa Clara, CA, USA) at Swegene Microarray Resource Centre (Lund, Sweden). Briefly, after a quality control of the RNA with Agilent Bioanalyzer 2100 (Agilent®), double stranded cDNA was generated by reverse transcription from 1 μg of total RNA. The cDNA was used as a template in an in vitro transcription reaction to generate amplified amounts of biotin-labelled cRNA.

After fragmentation of the cRNA, 15 µg cRNA in 200 µl hybridisation cocktail was hybridised onto Affymetrix MOE430 2.0 microarray chip for 16 hours at 45˚C. For standard post-hybridisation wash, double-staining and scanning protocols, Affymetrix GeneChip® Fluidics Station 400 and GeneChip® Scanner 3000 were used, respectively.

4.3. Microarray data analysis

We used the dChip software (Li and Wong, 2001a) for the analysis of microarray raw-data. After array normalization the model-based expression values were calculated using the PM-only model (Li and Wong, 2001b). Prior to the hierarchical clustering of samples, the filter genes function was applied to filter out probe sets with low expression level and low variation across samples. The variation across samples criterion was 0.25 < (Standard deviation / Mean) < 10, and the expression level had to be > 50 in all samples.

We used the compare function in dChip to identify differentially expressed genes between any two conditions. A gene was considered differentially expressed if it satisfied the following criteria: (i) a fold change >2, (ii) a p-value <0.05 by t-testing, and (iii) a present call in all R11 replicates when computing the 1c6 down-regulated genes or a present call in all 1c6 replicates when computing the 1c6 up-regulated genes. For each comparison the False Discovery Rate (FDR) was assessed by 200 random permutations of the samples. In all cases, the median FDR of 200 permutations was 0%.

We used the online Expression Analysis Systematic Explorer (EASE) software for the overrepresented biological theme analysis (Hosack et al., 2003; http://david.niaid.nih.gov/david/upload.asp). In order to make an EASE analysis for a

set of differentially expressed genes the Affymetrix probe set IDs were translated to a list of non-redundant GeneIDs (formerly LocusLink). Some IDs were not associated to a GeneID and those were not considered in the EASE analysis. The LocusLink-mouse dataset was used as background. When a GeneID was not found in the background, that GeneID was removed from the EASE analysis. In all EASE analyses, an EASE score of <0.05 was considered significantly overrepresented.

Affymetrix online NetAffx (http://www.affymetrix.com/analysis/index.affx) was used to annotate all gene lists.

4.4. Reverse transcription and polymerase chain reaction

Total RNA from developing R11, 1c3 and 1c6 EBs at day 0 and day 4 were subjected to first strand cDNA synthesis using Superscript™ II RT (Invitrogen). cDNA was amplified using *Taq* DNA Polymerase reaction mix (Fermentas) with the following primer pairs added: Bmper: Fw- GCCTGGGATTACCTGCTGC and Rev- ACACATTATGCAAGGGTTGTCTG; Dab2: Fw- CAATCTCAACTTCTTTCCCACCC and Rev- ATCAGTATCACCGTTCAGGGG; Dkk1: Fw- AAACCTTGGTAATGACCACAACG and Rev- AGAAGTGTCTTGCACAACACA; Flrt3: Fw- AGCGCAGGAGTGATCTGAA and Rev- TGGAAAGAGAAGGAAGCACAC; Gata6: Fw- CTCAGGGGTAGGGGCATCA and Rev- GAGGACAGACTGACACCTATGTA; Mest: Fw- GTGGTGGGTCCAAGTAGGG and Rev- AAGCACAACTATCTCAGGGCT; Nostrin: Fw- GTCAGGCTCAGCAAAGCACTA and Rev- TGCCAAGTTTTTGATGGAGGT; Nr5a2: Fw- TGAGGAACAACTCCGGGAAAA and Rev- CAGACACTTTATCGCCACACA; Pdgfra: Fw-

TCCATGCTAGACTCAGAAGTCA and Rev- TCCCGGTGGACACAATTTTTC; 1110032E23Rik: Fw- TTCTGTGACGTTAGCACACATT and Rev- GCAGATTCAGGAAACTCTCGGAT; Sox7: Fw- ATGCTGGGAAAGTCATGGAAG and Rev- CGTGTTCTGGTCACGAGAGA; Trps1: Fw- CTCTTCACGGCCTGCTTTAC and Rev- ATTTTATTCGGGTGCGTCTG ; Vegfc: Fw- AGCCAACAGGGAATTTGATG and Rev- CACAGCGGCATACTTCTTCA; Rps18: FW- GGGCTGGAGAACTCACGGAGGAT and Rev- GGCCCAGAGACTCATTTCTTCTT.

Annealing temperatures and the number of cycles used for RT-PCR are available upon request.

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Figure legends

Figure 1. Experimental design. R11 (wild type) and 1c6 (dnFGFR2) ES cell were cultured in three independent replicates (A). ES cells were cultured on cell culture plates with STO fibroblasts and LIF (B). The transfer of ES cells to Petri dishes without fibroblasts and LIF was denominated as day 0 of the EB culture. After 2 hours of transfer, the first cell population was harvested, thereafter EBs were harvested after 24, 48 and 96 hours of EB culture. Representative light microscope pictures show the morphology of R11 and 1c6 EBs during four days of development (C). RNA was isolated from EBs and subsequent hybridization to Affymetrix MOE430 2.0 mouse GeneChip was completed at Swegene microarray facility (D).

Figure 2. Hierarchical clustering of samples. Before clustering, a filter was applied to exclude probe sets with low expression and low variation across samples. Out of 45101 probe sets, 3938 remained after filtration. R11 is wild type and 1c6 is the dominant negative FGFR2 mutant. The last digit represents the day and the second last represent the replicate. Thus, R11_1.4 is wild type EB replicate 1 at day 4. The samples divided into two major clusters: A and B. Samples in group B (R11 day 1-4) clustered the biological replicates of each time point into separate groups. The group A samples were further divided into one cluster of all the day 0 samples (C) and one cluster of the 1c6 day 1-4 samples (D). Samples in group C also clustered the biological replicates into separate groups. However, in group D, 1c6 day 1 clustered the biological replicates into a separate group whereas 1c6 day 2 and 4 where mixed.

Figure 3. RT-PCR analysis. Total RNA was amplified from R11 and 1c6 EBs at day 0 and day 4. Randomly selected genes from Tables 1-4 were subjected to RT-PCR analysis where Rps18 was used as control.

Supplementary figure legends

Supplementary Figure 1. RT-PCR analysis. Total RNA was amplified from R11 and 1c3 EBs at day 0 and day 4. Note that similar gene expression was found in the 1c6 ES cell clone.

Supplementary tables

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Table 1

Top 20 down-regulated and top 10 up-regulated genes in 1c6 (dnFGFR2) embryoid bodies at day 0

Gene Title	Gene Symbol	Entrez Gene	Fold Change
Down-regulated			
SRY-box containing gene 17	Sox17	20671	-32.24
Proteoglycan 1, secretory granule	Prg1	19073	-18.78
Fibronectin leucine rich transmembrane protein 3	Flrt3	71436	-13.36
Forkhead box Q1 (Foxq1), mRNA	Foxq1	15220	-10.37
Laminin, alpha 1	Lama1	16772	-10.14
GATA binding protein 6	Gata6	14465	-9.58
Procollagen, type IV, alpha 1	Col4a1	12826	-8.36
Forkhead box A2	Foxa2	15376	-8.17
Laminin B1 subunit 1	Lamb1-1	16777	-7.63
RIKEN cDNA 1110032E23 gene	1110032E23Rik	68659	-6.55
Serine peptidase inhibitor, Kazal type 3	Spink3	20730	-6.53
Procollagen, type IV, alpha 2	Col4a2	12827	-6.51
GLI pathogenesis-related 1 (glioma)	Glipr1	73690	-6.06
Cathepsin C	Ctsc	13032	-5.75
Maelstrom homolog (Drosophila)	Mael	98558	-5.52

Similar to Eukaryotic translation initiation factor 1A (eIF-1A) (eIF-4C)	MGC107533	266459	-5.41
Carbonic anhydrase 2	Car2	12349	-5.40
EF-hand domain (C-terminal) containing 2	Efhc2	74405	-5.40
RIKEN cDNA 1200002N14 gene	1200002N14Rik	71712	-5.34
Gap junction membrane channel protein alpha 1	Gja1	14609	-5.32
Up-regulated			
Kelch repeat and BTB (POZ) domain containing 11	Kbtbd11	74901	4.62
Mesoderm specific transcript	Mest	17294	4.09
Neuronatin	Nnat	18111	3.97
Carboxypeptidase E	Cpe	12876	3.88
Secreted frizzled-related sequence protein 1	Sfrp1	20377	3.47
Left-right determination factor 2	Lefty2	320202	3.40
RIKEN cDNA 4930422N03 gene	4930422N03Rik	76871	3.18
G protein-coupled receptor 27 (Gpr27), mRNA	Gpr27	14761	3.01
Hedgehog-interacting protein	Hhip	15245	2.87
Glutamic pyruvate transaminase (alanine aminotransferase) 2	Gpt2	108682	2.68

Table 2

Top 20 down-regulated and top 10 up-regulated genes in 1c6 (dnFGFR2) embryoid bodies at day 1

Gene Title	Gene Symbol	Entrez Gene	Fold Change
Down-regulated			
Disabled homolog 2 (Drosophila)	Dab2	13132	-24.37
Proteoglycan 1, secretory granule	Prg1	19073	-24.17
SRY-box containing gene 17	Sox17	20671	-21.04
Transcription factor EC	Tcfec	21426	-18.55
Nitric oxide synthase trafficker	LOC329416	329416	-15.61
RIKEN cDNA 1200002N14 gene	1200002N14Rik	71712	-11.56
BMP-binding endothelial regulator	Bmper	73230	-11.02
Forkhead box Q1 (Foxq1), mRNA	Foxq1	15220	-10.68
Growth factor receptor bound protein 10	Grb10	14783	-10.41
GATA binding protein 6	Gata6	14465	-10.24
SRY-box containing gene 7	Sox7	20680	-9.97
Chloride intracellular channel 6	Clic6	209195	-9.00
Procollagen, type IV, alpha 1	Col4a1	12826	-8.48
Laminin B1 subunit 1	Lamb1-1	16777	-8.46
Platelet derived growth factor receptor, alpha polypeptide	Pdgfra	18595	-7.33

RIKEN cDNA 1110032E23 gene	1110032E23Rik	68659	-7.00
Parathyroid hormone receptor 1	Pthr1	19228	-6.61
Forkhead box A2	Foxa2	15376	-6.45
RIKEN cDNA 4933402E13 gene	4933402E13Rik	74437	-6.22
Tetraspanin 8	Tspan8	216350	-6.19
Up-regulated			
Neuronatin	Nnat	18111	4.79
RIKEN cDNA 4930422N03 gene	4930422N03Rik	76871	3.95
Kelch repeat and BTB (POZ) domain containing 11	Kbtbd11	74901	3.55
Mesoderm specific transcript	Mest	17294	3.54
Myosin IF	Myo1f	17916	3.35
Secreted frizzled-related sequence protein 1	Sfrp1	20377	3.13
Muscle and microspikes RAS	Mras	17532	3.08
ODZ3 (Odz3)	Odz3	23965	2.93
Carboxypeptidase E	Cpe	12876	2.87
G protein-coupled receptor 27 (Gpr27), mRNA	Gpr27	14761	2.86

Table 3

Top 20 down-regulated and top 10 up-regulated genes in 1c6 (dnFGFR2) embryoid bodies at day 2

Gene Title	Gene Symbol	Entrez Gene	Fold Change
Down-regulated			
SRY-box containing gene 17	Sox17	20671	-25.68
Dickkopf homolog 1 (Xenopus laevis)	Dkk1	13380	-19.35
Transthyretin	Ttr	22139	-19.13
Cubilin (intrinsic factor-cobalamin receptor)	Cubn	65969	-18.91
Amnionless	Amn	93835	-17.87
Transcription factor EC	Tcfec	21426	-17.51
SRY-box containing gene 7	Sox7	20680	-17.47
Laminin, alpha 1	Lama1	16772	-16.92
RIKEN cDNA 4933402E13 gene	4933402E13Rik	74437	-16.79
Fibronectin leucine rich transmembrane protein 3	Flrt3	71436	-16.74
Cathepsin H	Ctsh	13036	-15.72
Chloride intracellular channel 6	Clic6	209195	-15.04
Disabled homolog 2 (Drosophila)	Dab2	13132	-14.81
Nitric oxide synthase trafficker	LOC329416	329416	-14.02
Pepsinogen 5, group I	Pga5	58803	-13.77

Serine peptidase inhibitor, Kazal type 3	Spink3	20730	-13.54
Angiomotin	Amot	27494	-13.34
Growth factor receptor bound protein 10	Grb10	14783	-13.24
Proteoglycan 1, secretory granule	Prg1	19073	-13.08
Carbonic anhydrase 4	Car4	12351	-12.85
Up-regulated			
Neuronatin	Nnat	18111	5.57
Mesoderm specific transcript	Mest	17294	4.54
Myosin IF	Myo1f	17916	4.46
Trichorhinophalangeal syndrome I (human)	Trps1	83925	4.39
Kelch repeat and BTB (POZ) domain containing 11	Kbtbd11	74901	3.75
Vascular endothelial growth factor C	Vegfc	22341	3.38
RIKEN cDNA 4930422N03 gene	4930422N03Rik	76871	3.38
Inhibin beta-B	Inhbb	16324	3.11
Odd Oz/ten-m homolog 3 (Drosophila)	Odz3	23965	3.08
Hypothetical LOC433110	LOC433110	433110	3.05

Table 4

Top 20 down-regulated and top 10 up-regulated genes in 1c6 (dnFGFR2) embryoid bodies at day 4

Gene Title	Gene Symbol	Entrez Gene	Fold Change
Down-regulated			
S100 calcium binding protein G	S100g	12309	-24.42
SRY-box containing gene 17	Sox17	20671	-18.66
Laminin, alpha 1	Lama1	16772	-18.34
GATA binding protein 6	Gata6	14465	-18.06
Fibronectin leucine rich transmembrane protein 3	Flrt3	71436	-17.04
Aquaporin 8	Aqp8	11833	-16.52
Platelet derived growth factor receptor, alpha polypeptide	Pdgfra	18595	-15.81
SRY-box containing gene 7	Sox7	20680	-15.25
Alpha fetoprotein	Afp	11576	-14.75
Pepsinogen 5, group I	Pga5	58803	-14.59
Growth factor receptor bound protein 10	Grb10	14783	-14.48
Glutamyl aminopeptidase	Enpep	13809	-14.14
Cubilin (intrinsic factor-cobalamin receptor)	Cubn	65969	-14.10
Forkhead box A2	Foxa2	15376	-13.59

Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 1	Cited1	12705	-13.14
RIKEN cDNA 4933402E13 gene	4933402E13Rik	74437	-12.82
Klotho beta	Klb	83379	-12.60
Cathepsin H	Ctsh	13036	-12.54
Lectin, galactose-binding, soluble 2	Lgals2	107753	-12.04
Forkhead box Q1 (Foxq1), mRNA	Foxq1	15220	-11.98
Up-regulated			
Vascular endothelial growth factor C	Vegfc	22341	6.44
Myosin IF	Myo1f	17916	5.86
Mesoderm specific transcript	Mest	17294	5.58
cDNA sequence BC038881	BC038881	330671	5.29
Nuclear receptor subfamily 5, group A, member 2	Nr5a2	26424	5.19
Hypothetical LOC433110	LOC433110	433110	5.09
Neuronatin	Nnat	18111	4.93
RIKEN cDNA 4930422N03 gene	4930422N03Rik	76871	4.91
Procollagen C-endopeptidase enhancer protein	Pcolce	18542	4.64
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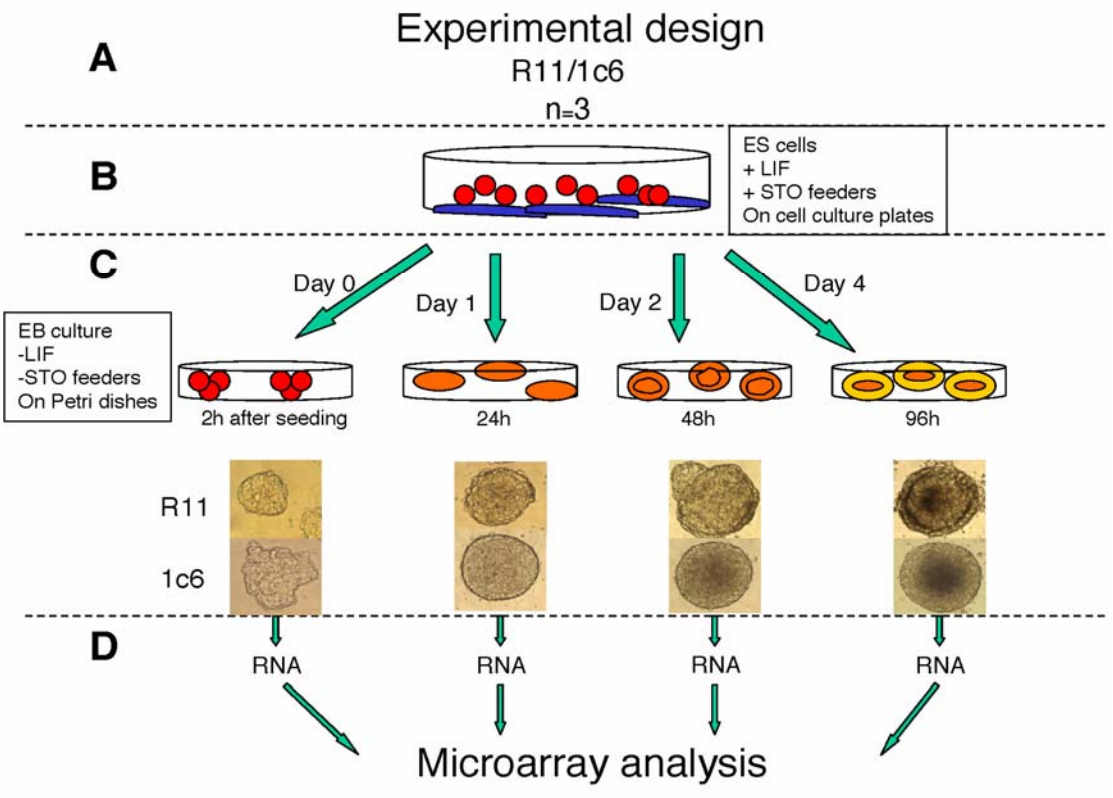
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-STO feeders
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96h
R11
1c6
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RNA
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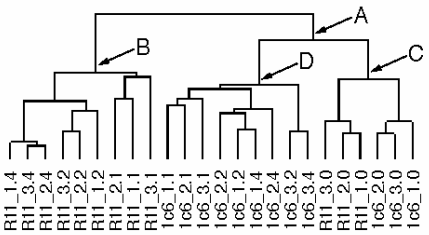
Microarray analysis

Figure 1

Figure 2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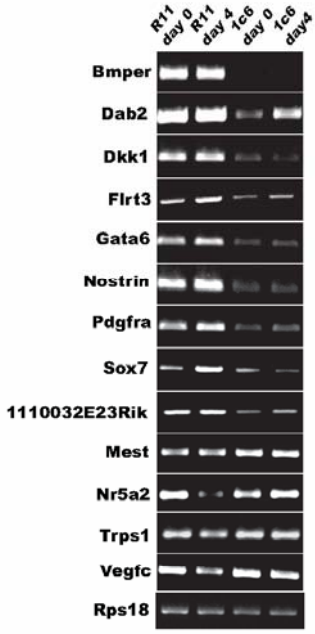
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Mest
Nr5a2
Trps1
Vegfc
Rps18

Figure 3

Supplement Figure 1