GDF11 induces differentiation and apoptosis and inhibits migration of C17.2 neural stem cells via modulating MAPK signaling pathway

Zongkui Wang [1] , Miaomiao Dou [1] , Fengjuan Liu [1] , Peng Jiang [1] , Shengliang Ye [1] , Li Ma [1] , Haijun Cao [1] , Xi Du [1] , Pan Sun [1] , Na Su [1] , Fangzhao Lin [1] , Rong Zhang [Corresp., 1] , Changqing Li [Corresp. 1]

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GDF11, a member of TGF-β superfamily, has recently received widespread attention as a novel anti-ageing/rejuvenation factor to reverse age-related dysfunctions in heart and skeletal muscle, and to induce angiogenesis and neurogenesis. However, these positive effects of GDF11 were challenged by several other studies. Furthermore, the mechanism is still not well understood. In the present study, we evaluated the effects of GDF11 on C17.2 neural stem cells. GDF11 induced differentiation and apoptosis, and suppressed migration of C17.2 neural stem cells. Besides, GDF11 slightly increased cell viability after 24h treatment, showed no effects on proliferation for about 10 days of cultivation, and slightly decreased cumulative population doubling for long-term treatment (p<0.05). Phospho-proteome profiling array displayed that GDF11 significantly increased the phosphorylation of 13 serine/threonine kinases (p<0.01), including p-p38, p-ERK and p-Akt, in C17.2 cells, which implied the activation of MAPK pathway. Western blot validated that the results of phospho-proteome profiling array were reliable. Based on functional analysis, we demonstrated that the differentially expressed proteins were mainly involved in signal transduction which was implicated in cellular behavior. Collectively, our findings suggest that, for neurogenesis, GDF11 might not be the desired rejuvenation factor, but a potential target for pharmacological blockade.

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43 **Abstract:**

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45 novel anti-ageing/rejuvenation factor to reverse age-related dysfunctions in heart and skeletal

46 muscle, and to induce angiogenesis and neurogenesis. However, these positive effects of GDF11

47 were challenged by several other studies. Furthermore, the mechanism is still not well

48 understood. In the present study, we evaluated the effects of GDF11 on C17.2 neural stem cells.

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50 cells. Besides, GDF11 slightly increased cell viability after 24h treatment, showed no effects on

51 proliferation for about 10 days of cultivation, and slightly decreased cumulative population

52 doubling for long-term treatment ($p < 0.05$). Phospho-proteome profiling array displayed that

53 GDF11 significantly increased the phosphorylation of 13 serine/threonine kinases ($p < 0.01$),

54 including p-p38, p-ERK and p-Akt, in C17.2 cells, which implied the activation of MAPK

55 pathway. Western blot validated that the results of phospho-proteome profiling array were

56 reliable. Based on functional analysis, we demonstrated that the differentially expressed proteins

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59 rejuvenation factor, but a potential target for pharmacological blockade.

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1. Introduction

Growth differentiation factor 11 (GDF11), also known as bone morphogenetic protein 11 (BMP11), is a secreted glycoprotein belonging to the transforming growth factor β (TGF-β) superfamily (Pepinsky et al. 2017; Walker et al. 2016). GDF11 plays an important role in anterior/posterior axial patterning during embryonic development (Oh et al. 2002). Similar to the negative effects of myostatin (also known as GDF8), which is 90% homology with GDF11 (Walker et al. 2017), in skeletal muscle, GDF11 acts as a negative regulator of neurogenesis in the olfactory epithelium (Wu et al. 2003) and in the developing spinal cord (Santos et al. 2012).

Recently, Loffredo et al. (2013) suggested that GDF11 was the rejuvenation factor to reverse age-related dysfunction in heart. Subsequently, it was confirmed that GDF11 repaired injured skeletal muscle and improved cognitive function (Katsimpardi et al. 2014; Sinha et al. 2014). However, the rejuvenating effects of GDF11 on heart, skeletal muscle and brain were questioned by a couple of independent studies. Egerman and colleagues (2015) showed that GDF11 supplementation inhibited muscle regeneration and decreased satellite cell expansion in mice, and suggested that GDF11 was not a rejuvenation factor but a potential target for pharmacologic blockade to treat age-related diseases. Hinken et al. (2016) also suggested GDF11 wasn't a rejuvenator of aged murine skeletal muscle satellite cells. In addition, others reported restoring GDF11 in old mice had no effect on cardiac structure or function (Smith et al. 2015). These conflicting studies offer compelling evidence that the effects of GDF11 are contradictory and demonstrate that the effects of GDF11 on neurogenesis are still not completely understood. Therefore, we require an in-depth knowledge of the effects and potential mechanism of action of GDF11 on regulating neural stem cells.

In the present study, we focused on the effects of GDF11 on the cellular behavior of C17.2 neural stem cells (including viability, proliferation, differentiation, apoptosis and migration), the changes in the phospho-proteome and the corresponding signaling pathways. We herein showed that GDF11 promoted differentiation and apoptosis, and suppressed migration of C17.2 cells. Besides, GDF11 stimulated cellular proliferation in a short time (within 24h), whereas high concentrations of GDF11 inhibited proliferation in a long-term cultivation (~ 20 days). Pathway-oriented proteome profiling revealed that GDF11 stimulation significantly activated phosphorylation of 15 proteins, including Smad2/3, Erk1/2, Akt1/2/3, p38, p70S6k, GSK-3α/3β and HSP27, which were mainly involved in MAPK signaling pathway. These data demonstrated the effects of GDF11 on neural stem cell (inducing differentiation and apoptosis, and suppressing migration) through the MAPK pathway.

2. Materials and methods

2.1 Agents

C17.2 neural stem cell line was purchased from zqxzbio (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM; catalog No. SH30022.01), Penicillin-Streptomycin Solution (catalog No. SV30010) and Trypsin (catalog No. SH30236.01) were obtained from Hyclone (Logan, Utah, USA). Fetal bovine serum (FBS; catalog No. 04-121-1A-US) and horse serum (HS; catalog No. 04-124-1A) were purchased from Biological Industries (Beit Haemek, Israel). Recombinant human/mouse/rat GDF-11/BMP-11 (catalog No. 1958-GD-010) and Phospho-MAPK proteome profiler array kit (catalog No. ARY002B) were obtained from R&D systems (Minneapolis, USA). Dimethyl sulfoxide (DMSO; catalog No. D2650) were purchased from Sigma-Aldrich (St. Louis, USA). NuPAGE LDS loading buffer (catalog No. NP0007) and NuPAGE 4-12% Bis-Tris gel (catalog No. NP0321BOX) were obtained from Invitrogen (Waltham, USA). Phosphatase inhibitor cocktail (catalog No.HY-K0022) and protease inhibitor cocktail (catalog No. HY-K0010) were from MedChem Express (Shanghai, China). RIPA buffer (catalog No. 89900) was from Pierce (Rockford, USA). Rabbit anti-Smad2/3 (catalog No. 8685S), rabbit anti-p-smad2/Smad3 (catalog No. 8828S) , rabbit anti-CREB (catalog No. 9197S), rabbit anti-p-CREB (catalog No. 9198S), rabbit anti-ERK (catalog No.12950S), rabbit anti-p-ERK (catalog No.4377T), rabbit anti-p38 (catalog No.8690S), rabbit anti-p-p38 (catalog No.4511T), mouse anti-nestin (catalog No.33475S), rabbit anti-βIII-tubulin (catalog No.5568S), rabbit anti-GFAP (catalog No.12389S), rabbit anti-β-actin (catalog No.4970S), rabbit anti-GAPDH (catalog No.2118S) and goat anti-rabbit IgG (catalog No.7074P2) were obtained from Cell Signal Technology (Beverly, MA, USA). Enhanced Cell Counting Kit-8 (CCK-8; catalog No.C0042) and BCA Protein Assay Kit (catalog No.P0010) were obtained from Beyotime Biotechnology (Beijing, China). Enhanced chemiluminescence (ECL; catalog No.32109) was form Pierce (Rockford, USA).

2.2 C17.2 cell culture

C17.2 cells were cultured on 25-cm^2 culture flasks in complete medium (DMEM supplemented with 10% (v/v) FBS, 5% (v/v) HS, 100 U/ml penicillin and 100 μg/ml streptomycin) at 37 ºC, 5% CO^2 in a humidified atmosphere. Media were changed every 2–3 days. After reaching 70-80% confluence, C17.2 cells were trypsinized and re-seeded at a density of 4*10^4 cells/mL in complete medium which was changed to starved medium (DMEM supplemented with 0.5% HS and 1% FBS) one day post seeding. After 6 h of serum starvation, different concentrations of GDF11 (0, 12.5, 25, 50 and 100ng/mL) were added, respectively.

2.3 Cell morphology analysis

C17.2 cells were seeded onto the 24-well plates at a density of 4*10^4 cells/well in 0.1 mL complete medium. After adherence, complete medium was replaced with starved medium for 6h and then, various concentrations of GDF11 were introduced when appropriate. GDF11-untreated

154   cells were served as control.

155   The cell morphology and viability were examined using LIVE/DEAD® viability /
156   cytotoxicity kit (catalog No.L3224) for mammalian cells (Invitrogen, USA) according to
157   the manufacturer's instructions under inverted fluorescence microscope (AXIO, Zeiss, Jena,
158   Germany). The live cells were stained with calcein AM in green, and the dead cells were stained
159   with ethidium homodimer-1(EthD-1) in red.

2.4 Cell viability and proliferation assay

161   Cell viability was assessed by CCK-8 assay. Briefly, 10 μL of CCK-8 agent was added to each
162   well 2 h before the termination of the experiment. The optical density (OD) values at 450 nm
163   were determined using SpectraMax M2^e (Molecular Devices, Sunnyvale, USA). Then, by
164   comparing the absorbance of GDF11-treated and untreated cells, percentage viability was
165   calculated.

166   For proliferation assay, $1*10^4$/mL cells were seeded in 12-well plates in triplicates. When the
167   cell cultured to ~80% confluence (generally 3 days), cells were trypsinized and manually
168   counted using a haemocytometer. Cell population doubling (PD) was calculated using the
169   following formulae:

170   $(1) PD = \log2 (N/N_0)$,

171   where N_0 represents the number of cells seeded at the initial passage, N is the final number of
172   cells.

2.5 Apoptosis assay

174   To investigate the apoptosis-inducing effect of GDF11, we identified apoptotic and necrotic
175   cells by Annexin V-FITC and propidium iodide (PI) dual staining using FACScan flow
176   cytometry (Becton-Dickinson, USA). Approximately $1*10^5$ cells were analyzed in each
177   experimental group. The cell populations were distinguished according to their positioning of
178   quadrants: live cells (Annexin V−/PI−), early/primary apoptotic cells (Annexin V+/PI−),
179   late/secondary apoptotic cells (Annexin V+/PI+) and necrotic cells (Annexin V−/PI+).

2.6 Scratch wound healing assay

181   C17.2 cells were cultured with complete medium in a 48-well plate at a density of 5 ×
182   10^4 cells/well. After reaching ~80% confluence, a single uniform scratch was made by using a
183   200μL pipette tip along the center of each monolayer. The scratch was lightly washed with PBS
184   twice to remove the detached cells, and then starved medium supplemented with various
185   concentrations of GDF11 was added (0ng/mL, 12.5ng/mL, 25ng/mL, 50ng/mL and 100ng/mL,
186   respectively). The scratches were monitored at 0h, 12h and 36h after scratching by taking photos

187 with inverted microscope to measure the wound closure. The wound closures of various
188 treatments at different time points were calculated with Image J software.

2.7 RNA extraction and qRT-PCR analysis

190   C17.2 cells were cultured on 12-well plates at a density of $4*10^4$ cells per well under standard
191 conditions. Upon reaching 80% confluence, the complete medium was changed to starved
192 medium. After 6 h of serum starvation, plates were treated with either indicated concentrations of
193 GDF11 (25ng/mL, 50ng/mL and 100ng/mL, respectively) or vehicle in starved medium for 4h.
194 Total RNA was extracted from the cultured cells using TRIZOL reagent according to the
195 standard procedure. Total RNA (1μg) was reverse transcribed in a final volume of 20 μL in a
196 reaction containing random primers, using iScriptTM cDNA Synthesis kit (Bio-Rad, Hercules,
197 USA). qRT-PCR was done using the Quantitect SYBR Green PCR kit (Qiagen) with a ABI
198 StepOnePlus Real-time PCR system (Applied Biosystems, Foster City, USA). Relative

199 expression was calculated using the $2^{-\triangle\triangle Ct}$ method by normalizing with GAPDH housekeeping

200 gene expression and presented as fold changes relative to control. The primers for qRT-PCR
201 were synthesized by Beijing Genomics Institute (Shenzhen, China) and the details of primer
202 sequences are shown in Supplementary Table S1.

2.8 Phospho-proteome profiling array

204   Human phospho-MAPK array kit was used to determine the relative levels of phosphorylation
205 of mitogen-activated protein kinases (MAPKs) and other serine/threonine kinases with or
206 without GDF11 treatment. Briefly, C17.2 cells were rinsed with PBS and solubilized with Lysis
207 Buffer 6 (provided in Human Phospho-MAPK Array Kit) at $1*10^7$ cells/mL. After rocking
208 gently at 2-8°C for 30 min, the lysates were centrifuged at 14,000 g for 5 min, and the
209 supernatant was collected and detected the protein contents using BCA protein assay. The arrays
210 were blocked by Buffer 5 for 1h on a rocking platform shaker. Afterwards, the mixture of sample
211 and detection antibody cocktail were introduced and incubated overnight at 2-8°C on a rocking
212 platform shaker. The following day, the membranes were washed three times, and then were
213 incubated in streptavidin-HRP for 30 min followed by three washes. The protein blots were
214 developed by ECL reagents. Densitometry analysis was measured with the Quantity One
215 software, and the averaged intensity was calculated by subtracting the averaged background
216 signal. The fold change was obtained by comparing GDF11-treated samples with the untreated
217 control (indicated as a value of 1):

218   Fold change = average intensity$_{\text{(GDF11-treated)}}$ / average intensity$_{\text{(control)}}$.

219 The respective coordinates of all the antibodies on the arrays and the corresponding
220 phosphorylation sites can be found in "Supplementary Table S2".

2.9 Western Blot analysis and validation

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222   C17.2 cells were cultured in 6-well dishes in starved medium with or without GDF11 for 24h.
223   Then, the cells were lysed in RIPA buffer containing 1× phosphatase inhibitor cocktail and 1×
224   protease inhibitor cocktail on ice for 30 min, and centrifuged at 14000 g for 5 min at 4°C. The
225   supernatants were collected, and the protein concentration was determined by BCA protein assay
226   kit. The samples were mixed with 4× NuPAGE LDS loading buffer, separated on NuPAGE 4-12%
227   Bis-Tris gels, and subsequently transferred to PVDF membranes by a wet transfer apparatus
228   (Bio-Rad, Hercules, USA). Following blocking with superblock at room temperature for 2h, the
229   membranes were incubated with rabbit anti-β-actin (1:1000), anti-Smad2/3 (1:1000), anti-p-
230   Smad2/3 (1:1000), anti-CREB (1:1000), anti-p-CREB (1:1000), anti-ERK (1:1000), anti-p-ERK
231   (1:1000), anti-p38 (1:1000), anti-p-p38 (1:1000), anti-nestin (1:1000), anti-βIII-tubulin (1:1000),
232   anti-GFAP (1:1000) and anti-GAPDH   (1:1000), respectively, at 4°C overnight. After washing
233   with PBST (PBS supplemented with 0.1% Tween 20), membranes were incubated with
234   horseradish peroxidase-conjugated goat anti-rabbit IgG (1:3000) at room temperature for 2h, and
235   were visualized by ECL reagents. The ImageJ software was used for densitometric analyses of
236   the blots.

2.10 Bioinformatic analyses

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238   To further understand the functions and features of the identified and quantified proteins, we
239   annotated functions and features of proteins from several different categories,
240   including subcellular localization, domain, Gene Ontology (GO) and pathway.

241   WoLF PSORT (a subcellular localization predication tool, version of PSORT/PSORT II) and
242   SubLoc (http://www.bioinfo.tsinghua.edu.cn/SubLoc/) were used to predict subcellular
243   localization of all identified differentially expressed proteins.

244   The domain functional description of the differentially expressed proteins were annotated by
245   InterProScan (a sequence analysis application) based on protein sequence alignment method, and
246   the InterPro domain database was used (http://www.ebi.ac.uk/interpro/).

247   GO annotation was derived from the UniProt-GOA database (http://www.ebi.ac.uk/GOA/),
248   and the differentially expressed proteins were classified by GO annotation based on the three
249   categories (GO term level 1): biological process, cellular component and molecular function.
250   According to GO annotation information of the identified proteins, we summed up the amount of
251   the differentially expressed proteins in each GO term of level 2.

252   The protein-protein interaction networks and pathways were annotated by Kyoto Encyclopedia
253   of Genes and Genomes (KEGG) database.

2.11 Statistical analysis

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255   The results were presented as the mean ± standard error (SE). Multi-group comparisons were
256 performed by one-way ANOVA followed by Tukey's post hoc test. Paired analysis of control
257 and treatment was accomplished using two-tailed unpaired or unpaired Student's t –tests when
258 appropriate. In addition, Statistical analyses were conducted using SPSS statistics
259 software,version 17.0 (SPSS Inc., Chicago, USA), and $p < 0.05$ was considered statistically
260 significant..

261 3 Results

262 3.1 The positive and negative effects of GDF11 on cellular viability and

263 proliferation

264   When compared with the counts of C17.2 cells initially seeded, both GDF11- and vehicle-
265 treated cells significantly proliferated after 72h of cultivation (Supplementary Fig. S1). Imaging
266 revealed that GDF11 significantly altered the morphology of C17.2 cells (Fig.1a). Cells without
267 GDF11 treatment remained their native neural stem cell state (Fig.1a, control), whereas cells
268 treated with various concentrations of GDF11 showed visual outgrowth of neuritis, displaying
269 phenotypes similar to neuron- and astrocyte-like cells (Fig.1a, GDF11). Remarkably, compared
270 to the control, supplement with high concentrations of GDF11 (50 and 100 ng/mL) significantly
271 resulted in morphological changes (differentiation and apoptosis) (Supplementary Fig.S1a).

272   To investigate the effect of GDF11 on cell viability, C17.2 cells were treated with indicated
273 concentrations of GDF11 (0, 12.5, 25, 50 and 100 ng/mL) for a 72h period, followed by CCK-8
274 assays. GDF11 slightly increased (less than 10%, $p<0.05$) cell viability after 24h treatment,
275 whereas it did not affect the cell viability after 72h treatment (Fig.1c).

276   As displayed in Fig.1d, all groups of C17.2 cells showed robust proliferation for the 6-passage
277 duration. GDF11 showed no effect on C17.2 cell proliferation till the 4th passages. From the 5th
278 passage, the low concentrations of GDF11 (12.5 and 25 ng/mL) still didn't affect the
279 proliferation of C17.2 cells, whereas higher concentrations of GDF11 (50 and 100 ng/mL)
280 significantly inhibited cell proliferation ($p<0.05$) and the exposure of C17.2 cells to 100 ng/mL
281 GDF11 resulted in the lowest cumulative population doubling level during the 6 passages of
282 cultivation amongst the 5 groups, which was approximately 17% lower than control ($p<0.05$).

283   Next, we detected the mRNA expression of cyclin D1 and cyclin D2, the cell cycle-related
284 proteins. GDF11 slightly but not significantly attenuated the expression of cyclin D1 and cyclin
285 D2 in the mRNA levels (Fig. 2d; $p>0.05$). These provide a potential molecular basis for the
286 effects of GDF11 on C17.2 cell viability and proliferation.

287   Together, these results revealed that GDF11 slightly increased cell viability after a short-term
288 (24h) cultivation and showed no effect on cell viability from 1st to 4th passage of cultivation
289 (approximately 10 days), whereas high concentrations of GDF11 significantly suppressed

290 cumulative population doubling for a long-term treatment.

3.2 GDF11 induced differentiation and apoptosis of C17.2 cells

292 The mRNA levels of the neural progenitor cell marker, nestin, were noticeably decreased after
293 being treated with GDF11, as compared to control levels (Fig. 2a; p<0.01). By contrast, the
294 GDF11-treated groups showed significant increase in βIII-tubulin (neuronal biomarker) and
295 GFAP (astrocytic biomarker) mRNA expression as compared to the control (Fig. 2a; p<0.05).
296 These all indicated the maturation and differentiation of C17.2 neural stem cells.   The
297 differences in nestin mRNA expression among the groups of GDF11 treatment were, however,
298 not significant, similar to βIII-tubulin and GFAP. Concomitantly with the mRNA expression, the
299 protein levels of nestin, βIII-tubulin and GFAP confirmed the similar results by western blot (Fig.
300 2b and c). When compared with the control, GDF11-treated cells showed the protein level of
301 nestin was significantly attenuated whereas βIII-tubulin and GFAP were up-regulated (Fig. 2b
302 and c), further indicating that GDF11 induced neuronal and astrocytic differentiation. However,
303 no dose-dependent effect of GFD11 was observed.

304 The results of Annexin V-FITC/PI dual staining revealed that GDF11 substantially induced
305 apoptosis of C17.2 cells. As shown in Fig. 1b and e, the number of total (both early and late)
306 apoptotic cells significantly increased in a GDF11 dose-dependent manner.   After 72h of
307 cultivation, the apoptotic cells were negligible in C17.2 cells without GDF11-treated, whereas
308 there were 2.1%, 9.8%, 13.1% and 17.7% of cells exhibiting apoptosis as a result of exposure to
309 12.5, 25, 50 and 100ng/mL GDF11, respectively (p<0.05). Meanwhile, the amount of necrotic
310 cells showed a slight but significant increase when treated with GDF11.

3.3 GDF11 suppressed the migration of C17.2 cells

312 The migration of C17.2 cells was performed by a "scratch wound healing'' assay. The wound
313 closure data are shown in Fig.3. It was observed that the wound closure increased as cell
314 migration progressed over time. After 12 h, the wound area had little difference compared to the
315 initial scratch area. As compared with that of 0h, wound area of 36h significantly decreased,
316 displaying 25.1% (0 ng/mL GDF11), 64.9% (12.5 ng/mL GDF11), 60.4% (25 ng/mL GDF11),
317 70.9% (50 ng/mL GDF11) and 75.7% (100 ng/mL GDF11) wound area, respectively (Fig.3b).
318 These implied wound closure was significantly inhibited when cells were treated with GDF11.
319 Of note, it was revealed that GDF11 showed slight but significant dose-dependent effects in the
320 inhibition of the migration. Together, these results demonstrated that GDF11 significantly
321 suppressed (but not completely abolished) the migratory potential of C17.2 neural stem cells.

3.4 GDF11 activated phosphorylation levels of selected signaling kinases

323 We deduced that, in C17.2 cells, GDF11 transmitted signals through phosphorylation of
324 Smads, as GDF11 belongs to TGF-β superfamily. First of all, we analyzed the effects of GDF11

325 on TGF-β signal pathway (the classical pathway activated by TGF-β family members) in C17.2
326 neural stem cells. GDF11 showed no effect on both the mRNA and protein levels of Smad3
327 (Fig.2e and Fig.4a). For Smad2, GDF11 significantly up-regulated the transcriptional level other
328 than the protein level (Fig.2e and Fig.4a). As shown in Fig.4a, cells untreated with GDF11
329 (control) displayed negligible phosphorylation of Smad2/3. On the contrary, presence of GDF11
330 pronouncedly phosphorylated Smad2/3 (p<0.05). However, no dose-dependent effect of GDF11
331 on Smad2/3 phosphorylation was observed. Moreover, we investigated the mRNA levels of
332 receptors of TGF-β superfamily, activin type IIB receptor (ActRIIB) and the type I receptors,
333 activin receptor-like kinase 5 (ALK5). The results of qRT-PCR revealed that GDF11 didn't alter
334 mRNA expression of ActRIIB and ALK (Fig.2e).

335 In order to further research the signal pathways affected by GDF11, we compared the
336 phosphorylation levels of MAPKs in C17.2 cells treated with vehicle or GDF11 using a
337 phospho-MAPK array kit. The fold changes were calculated from the ratio of intensity of the
338 MAPK array from the GDF11 treated C17.2 cells to the control (untreated cells). Cut-off values
339 were set 1.5-fold for up-regulated expression and 0.67-fold for down-regulated expression of a
340 protein. We observed significant increases in the phosphorylation levels of several proteins in
341 GDF11-treated cells compared with the untreated cells (Fig.5, Supplementary Table S2 and
342 Supplementary Fig.S2). Overall, 50% (13/26) proteins showed significantly increased
343 phosphorylation after treatment with GDF11, whereas the phosphorylation levels of the
344 remaining 50% (13/26) proteins were still unchanged. Strikingly, when treated with GDF11,
345 there were no proteins that showed decreased phosphorylation. In addition, the differentially
346 expressed proteins that showed the most significant increases included Creb (3.42 times
347 increased), HSP27 (3.05 fold increased), Akt1/2/3 (2.55-, 2.47- and 1.50-fold increased
348 expression, respectively), GSK-3β and GSK3α/β (2.12- and1.50 -fold increased, respectively),
349 p38 α/β (3.21 and 1.73 times increased), Erk1 (1.57), MKK3/6 (2.03- and 1.52- fold increased,
350 respectively) and p70s6k (1.93 times increased) (Fig.5c and Supplementary Fig.S2; p<0.05).
351 These indicated that GDF11 activated the MAPK/Erk and p38 MAPK pathways but not JNK
352 pathway in C17.2 neural stem cells. Remarkably, many of these differentially expressed proteins
353 are involved in signal transductions of cell survival and apoptosis.

354 3.5 Functional Classification of Differentially Expressed Proteins

355 As shown in Table 1, the differentially expressed proteins were mainly classified as
356 cytoplasmic (n=8), nuclear (n=7) and mitochondrial (n=1) proteins.

357 For an overview of the differentially expressed proteins, GO annotation was carried out to
358 identify the significantly enriched GO functions. According to the analysis, the 15 differentially
359 expressed proteins between GDF11-treated cells and control were mainly clustered into 38
360 functional groups, including 18 biological processes, 12 cellular components, and 8 molecular
361 functions (Fig.6a).

362 Biological process category according to GO annotations indicated that all the 15 differentially

363 expressed proteins were involved in metabolic process. And other significant function groups
364 included cellular process (13/15), response to stimulus (13/15), signaling (13/15) and localization
365 (12/15), etc (Fig.6a).

366   In the category of cellular components, the differentially expressed proteins were mainly
367 involved in cell (13/15), organelle (14/15), cytoplasm (12/15) and nucleus (13/15), indicating the
368 similar subcellular localization that was obtained from WoLF PSORT (Table 1). Not only the
369 similarities but also differences were found between the cellular component category and
370 subcellular localization results. According to the functional analysis of GO annotation, we found
371 six proteins were involved in plasma membrane, however, no membrane-associated proteins
372 were observed from subcellular localization results.

373   The most representative molecular function category was "binding", which accounted for all
374 the 15 differentially expressed proteins, and most of the differentially expressed proteins were
375 also involved in catalytic activity (11/15), transferase activity (11/15) and kinase activity (11/15).
376 These results also elucidated that the related signal pathways activated by GDF11.

3.6 KEGG Enriched Pathways

378   To explore the potential mechanisms for GDF11-mediated cell behavior (cellular proliferation,
379 differentiation, apoptosis and migration) in C17.2 neural stem cells, we used the KEGG database
380 to determine the protein-protein interaction networks and pathways involved in the up-regulated
381 phosphoproteins. The 15 differentially expressed proteins were mainly mapped to 51 pathways
382 according to the KEGG database, which were mainly associated with environmental information
383 processing (signal transduction), organismal systems (immune system, nervous system,
384 endocrine system and ageing), cellular processes (cell growth and death, transport and
385 catabolism, and cellular community) and human diseases (drug resistance, endocrine and
386 metabolic diseases, neurodegenerative diseases, infectious diseases, and cancers)
387 (Supplementary Table S3). These all indicated the differentially expressed proteins were mainly
388 involved in signal transduction of cellular behavior. Furthermore, domain functional description
389 of the differentially expressed proteins annotated by InterProScan, were significantly enriched in
390 protein kinase domain (25.58%) and protein kinase-like domain (25.58%) (Fig.6b). These were
391 also in line with the results of molecular function category of GO annotation which indicated the
392 differentially expressed proteins were mainly in connection with catalytic activity, transferase
393 activity and kinase activity (Fig. 6a).

394   A major overlapping network was enriched in this analysis (Fig.6c). Three canonical signaling
395 pathways (TGF-β, PI3K-Akt and MAPK signaling pathways), that were activated by the up-
396 regulation of phosphoproteins, were identified, and the cross-talking signaling cascade was
397 shown as well. One mainly functional cluster was apparent in the protein-protein interaction,
398 including Akt1/2/3 (the key components in the PI3K-Akt signal pathway), Erk1 (the key
399 components of MAPK/Erk pathway) and p38α/β (the key components in the p38 MAPK signal
400 pathway). These results provided a possible resource for future studies of the proteins involved

401 in GDF11-treated C17.2 cells.

3.7 Validation of selected differentially expressed proteins

403 To confirm the results of phospho-MAPK array, three differentially expressed protein
404 candidates (Creb, p38 and Erk) were selected for further validation using western blot. Total
405 protein lysates from C17.2 cells cultured with indicated concentrations of GDF11 (0, 12.5, 25, 50
406 and 100 ng/ml, respectively) were prepared and the phosphorylation levels were determined by
407 their respective phosphorylated antibodies. When compared with control, no detectable changes
408 in total Creb, p38 or Erk protein expression were observed in GDF11-treated C17.2 cells.
409 Nevertheless, GDF11-treatment significantly increased the phosphorylation levels of Creb, p38
410 and Erk (Fig.4b, c and d; all p <0.05). These western blot results were generally consistent with
411 the results of the phospho-MAPK array.

4 Discussion

413 Around the world, the number of aged people is precipitously increasing. Therefore, searching
414 for anti-ageing or rejuvenating factors is quite important to develop therapeutic strategies for the
415 treatment of age-related diseases.   Recently, GDF11 was suggested as a potential rejuvenating
416 factor, which not only reversed age-related cardiac hypertrophy and dysfunction in skeletal
417 muscle in mouse (Loffredo et al. 2013; Poggioli et al. 2016; Sinha et al. 2014), but also induced
418 rejuvenation of impairments in cognitive function of ageing mouse by remodeling the cerebral
419 vascular and enhancing neurogenesis (Katsimpardi et al. 2014). Recently, a prospective cohort
420 study also revealed that higher levels of GDF11/8 were associated with lower risk of
421 cardiovascular events and death in patients with stable ischaemic heart disease (Olson et al.
422 2015). However, these initial findings have been challenged by later recent studies. It was
423 reported that GDF11 inhibited muscle regeneration and decreased satellite cell expansion in mice
424 (Egerman et.al. 2015). Hinken et al. (2016) also suggested GDF11 wasn't a rejuvenator for aged
425 murine skeletal muscle satellite cells. In addition, restoring GDF11 in old mice showed no effect
426 on pathological hypertrophy (Smith et al. 2015).

427 Because GDF11 was reported to improve neurogenic rejuvenation, we hypothesized that
428 GDF11 influenced the cellular behavior of C17.2 neural stem cells, including viability,
429 proliferation, differentiation, apoptosis and migration. Therefore, we focused on the effects and
430 potential mechanism of action of GDF11 on viability, proliferation, differentiation, apoptosis and
431 migration in C17.2 cells. Here, our results indicated that GDF11 substantially induced
432 differentiation and apoptosis, and suppressed migration of C17.2 cells mainly through MAPK
433 signal pathway. Meanwhile, GDF11 induced sight but significant increases in cellular viability in
434 a short time of growth (24h) and showed no effects on cellular viability for medium-term
435 cultivation (< 4 passages; approximately 10 days). For long-term cultivation (> 4 passages), high
436 concentrations of GDF11 significantly inhibited the proliferation of C17.2 cells (Fig. 1c and d;
437 p<0.05). To the best of our knowledge, we are unaware of any similar published results.

438   Strikingly, similar to our results, it was found that GDF11 slightly increased cell viability after
439   short-term treatment and slightly decreased cell viability after long-term treatment in human
440   umbilical vein endothelial cells (Zhang et al. 2016). Previously, it was suggested that GDF11
441   acted as a negative regulator of neurogenesis (Wu et al. 2003). Recently, Williams *et al*. (2013)
442   reported a controversial finding that GDF11 suppressed proliferation and migration of Cor-1
443   cells, whereas no effect on differentiation was observed. The conflicting results may be caused
444   by GDF11 from different vendors, different batches of GDF11 from the same manufacturer, or
445   cells from different sources.

446   As a member of TGF-β superfamily, it was reported that GDF11 activated TGF-β signal
447   pathway as a consequence of phosphorylating Smad2/3 in several cell types (Liu et al. 2016;
448   Loffredo et al. 2013; Zhang et al. 2016). In the present study, we successfully observed GDF11
449   phosphorylated Smad2/3 in C17.2 neural stem cells. Consistent with our results, it was also
450   confirmed that Cor-1 neural stem cell line was able to respond to GDF11 stimulation by
451   Smad2/3 phosphorylation (Williams et al. 2013). It is widely known that the Smad2/3-dependent
452   TGF-β signals have been implicated in the proliferation and differentiation of neural stem cell.
453   For example, GDF11 negatively regulated self-renewal of neuroepithelial stem cells through
454   TGF-β signals (Falk et al. 2008).

455   Proteins, not genes, are the specific practitioners of cellular life organisms. Although
456   genome and transcriptome analyses are very useful to reveal the mechanism of GDF11
457   stimulation, proteomic profiles may not be accurately predicted by transcriptome profiling due to
458   several factors, such as post-translational modifications. Therefore, research on proteomics is
459   helpful to provide new information concerning the C17.2 cells response to GDF11 stimulation.
460   Based on phospho-proteome profiling array and bioinformatic analysis (Figs.5 and 6), we found
461   15 differentially expressed proteins, including p38, Erk, Akt, GSK3α/β, Creb, MKK3/6, p70S6k
462   and HSP27, which were mainly involved in signal transductions of cell survival and apoptosis.
463   Besides TGF-β signal pathway, we also found Akt pathway and two important MAPK pathways
464   (Erk MAPK and p38 MAPK pathways) were activated, but not JNK pathway. Similarly, it also
465   reported that GDF11 activated TGF-β/Smad2/3 but   suppressed JNK signaling pathways in
466   apolipoprotein E-null mice (Mei et al. 2016). The functions of Erk MAPK and p38 MAPK
467   pathways are complex, which are involved in controlling cell proliferation, differentiation,
468   survival/apoptosis and migration (Wagner & Nebreda 2009). Various studies demonstrated Erk
469   MAPK pathway was involved in cellular proliferation and migration (Khodosevich 2009; Wu et
470   al. 2014). Although p38 MAPK pathway is normally associated with anti-proliferative and
471   apoptotic functions (Wagner & Nebreda 2009), it was also reported that p38 was implicated in
472   pro-survival functions, including positively regulating proliferation, differentiation and anti-
473   apoptosis (Halawani et al. 2004; Ricote et al. 2006; Terriente-Félix et al. 2017; Thornton et al.
474   2008). MAPK/Erk, Akt and p38 MAPK pathways were required for the migration of cortical
475   neurons upon HGF stimulation (Segarra et al. 2006), however, we observed GDF11 significantly
476   suppressed the migratory capacity of C17.2 neural stem cells with the activation of Erk MAPK,
477   PI3K/Akt and p38 MAPK pathways. Of the 15 differentially expressed proteins we identified,

478   HSP27 and p70S6K are two important downstream effectors of Akt pathway.
479   Mechanistically, PI3K/Akt phosphorylates HSP27 and p70S6K, which facilitate protein folding
480   and control protein synthesis, to inhibit apoptosis and promote proliferation (Khodosevich 2009;
481   Li et al. 2008; Rane et al. 2003). All of these suggested that GDF11 regulated the proliferation,
482   differentiation, apoptosis and migration of C17.2 cells by cross-talking with MAPK signaling
483   pathway.

484   Neuronal migration is a complex and key process in physiological and pathological conditions.
485   Increasing the quantity of nerve cells and the migration of neurons to the final position are
486   critical to reverse age-related dysfunction in brain (Contreras-Vallejos et al. 2012; Martino et al.
487   2011; Zhao et al. 2008). It should be noted that, although GDF11-treatment for 24h slightly
488   increased the viability of C17.2 cells (Fig.1c), it showed no effect on cell death (Fig. S1b).
489   GDF11 didn't change the   cell viability after 72h cultivation (Fig.1c), whereas it
490   significantly stimulated cell death (Fig.S1a). In addition, we found GDF11 significantly
491   suppressed the migration of C17.2 cells. Despite the fact that GDF11 indeed induced C17.2 cells
492   to differentiate into neurons and astrocytes, our point of view is that it should be cautious if
493   GDF11 is considered as a rejuvenated factor for neural stem cells.

494 5 Conclusion

495   In the present study, we found that GDF11 was an important regulator of neural stem cell. In
496   C17.2 neural stem cells, GDF11 showed a positive effect on cell viability after 24h treatment but
497   displayed a tendency of a negative effect for long-term cultivation. In addition, GDF11
498   significantly induced differentiation and apoptosis, and suppressed migration of C17.2 neural
499   stem cells. Further analysis of MAPK signaling pathway, which was activated by GDF11,
500   preliminary illustrated the potential mechanism of action by which the cellular behavior was
501   induced. Taken together our current findings implied that GDF11 might be a potential target for
502   pharmacologic blockade instead of a rejuvenated factor for neural stem cells.

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Figure 1

Effect of GDF11 on C17.2 cells.

(a) The representative images of live and dead cell staining. C17.2 cells were cultured with indicated concentrations of GDF11. Images were obtained at 200X magnification by inverted fluorescence microscope. The live cells were stained with calcein AM in green, and the dead cells were stained with EthD-1 in red. (b) GDF11 induced apoptosis in C17.2 neural stem cells. C17.2 cells were treated with vehicle or GDF11 (12.5, 25, 50 and 100ng/mL) for 48h and cell distribution was analysed using Annexin V-FITC and PI dual staining. The FITC and PI fluorescence was measured by flow cytometer with FL-1 and FL-2 filters, respectively. Lower left quadrant-- live cells (Annexin V−/PI−), lower right quadrant--early/primary apoptotic cells (Annexin V+/PI−), upper right quadrant--late/secondary apoptotic cells (Annexin V+/PI+) and upper left quadrant--necrotic cells (Annexin V−/PI+). (c) The viability of C17.2 cells after 24h or 72h of cultivation with various concentrations of GDF11 or vehicle was measured using CCK-8 method.. N=3, p<0.05. (d) Cumulative population doubling levels of C17.2 cells supplemented with different GDF11 concentrations for a total period of 6 passages. N=4, *p<0.05 compared with control. (e) Quantitative analyses of the GDF11 effect on apoptosis. N=3, *p<0.05versus with vehicle control and **p<0.01versus with vehicle control.

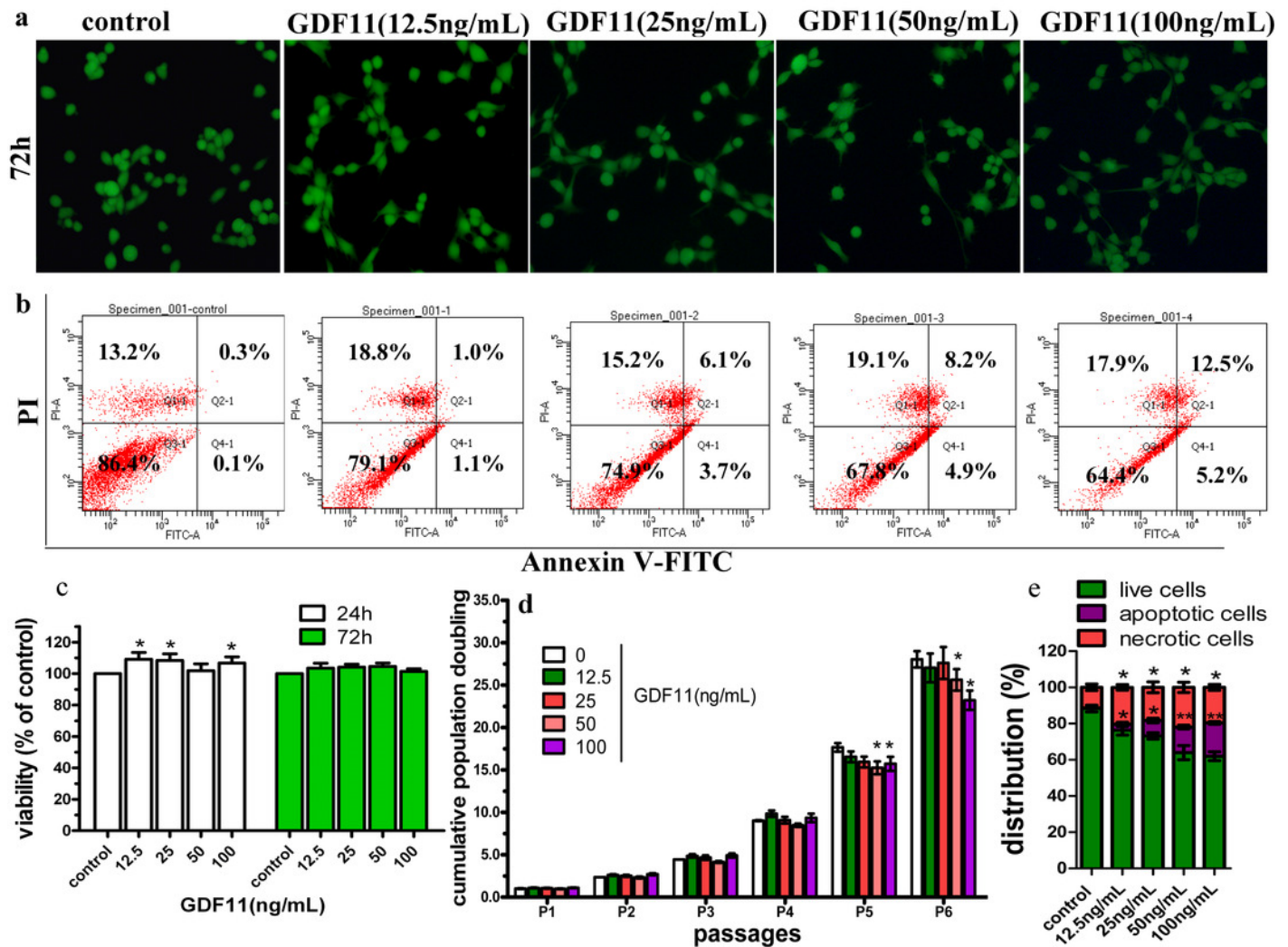



Figure 2

The effect of GDF11 on mRNA and protein expression

(a) Nestin, βIII-tubulin and GFAP mRNA levels of C17.2 cells after GDF11 or vehicle (control) treated for 5h.The results display mean ± SD of n= 4 and were analysed by one-way ANOVA followed by Tukey's post hoc test. *p< 0.05 as compared with mRNA levels in control cells. (b) Nestin, βIII-tubulin and GFAP protein levels of C17.2 cells after GDF11 (50ng/mL, "T") or vehicle ("C") treated for 72h. (c) Quantitative analyses of protein expression in relation to β-actin expression. Results were analysed by Student's t-test. N=6, *p< 0.05. (d) Cyclin D1, Cyclin D2 and EGFR mRNA expression after GDF11 or vehicle treated for 5h. N=5, *p< 0.05 and **p<0.01. (e) The mRNA levels of Smad2, Smad3, Alk5 and ActRIIB after GDF11 or vehicle treated for 5h. N=5, *p< 0.05 and **p<0.01.

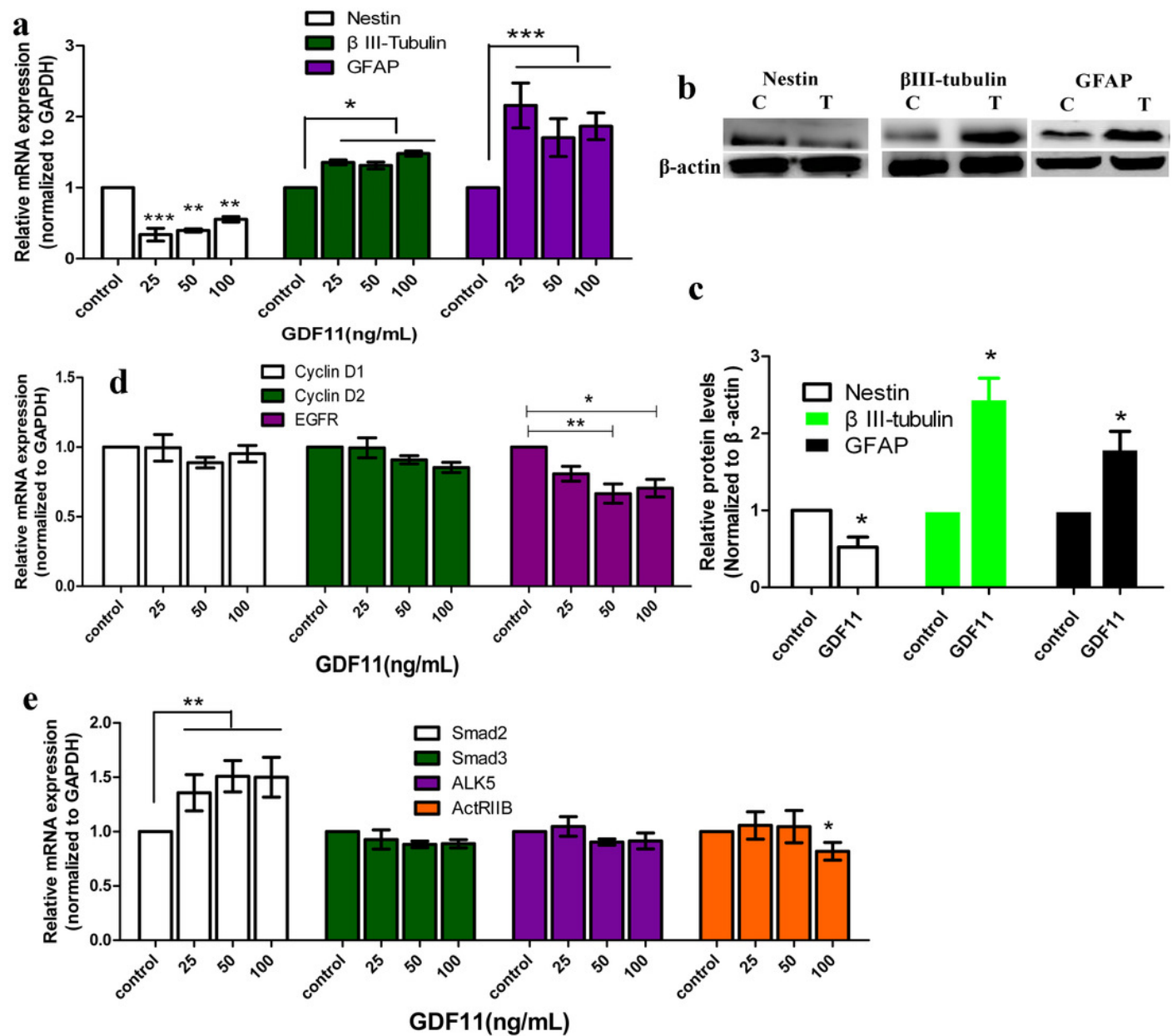



Figure 4

GDF11 increased the phosphorylations of Smad2/3, Creb, p38 and Erk in C17.2 neural stem cells.

(a-d). After 24h cultivation, GDF11 showed no effect on total protein of Smad2/3, Creb, p38 and Erk, but significantly phosphorylated Smad2 (Ser465/467), Smad3(Ser423/425), Creb(Ser133), p38(Thr180/Tyr182) and Erk (Thr202/Tyr204) in C17.2 cells. N=4, *p< 0.05, **p<0.01 and ***p<0.001 when compared with control.

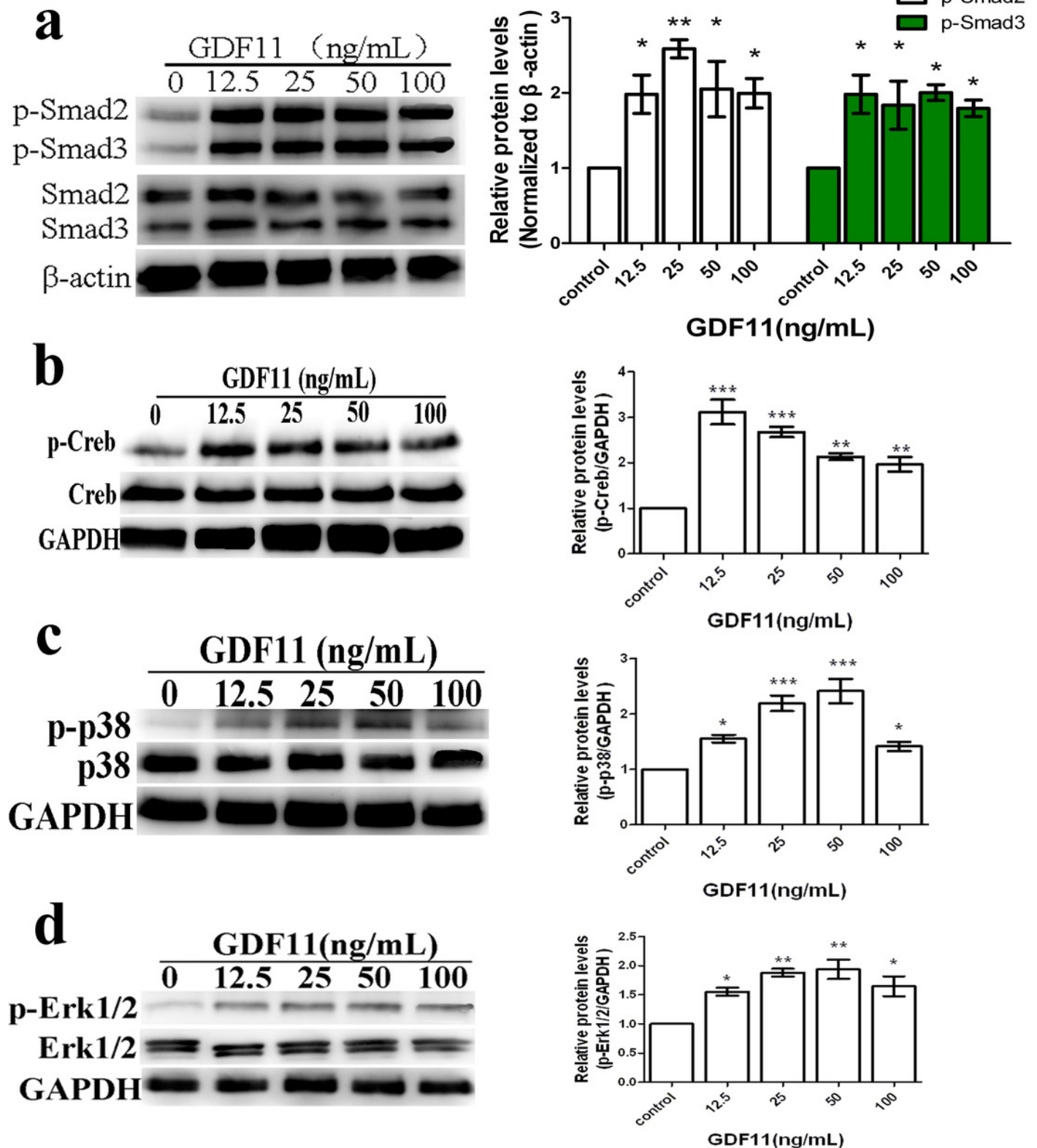



Figure 5

Alterations of MAPK pathway-related proteins in GDF11-treated C17.2 cells.

(a). Phosphoproteome profiling of C17.2 cells in response to GDF11 stimulation. Total cell lysates from C17.2 cells with25ng/mL GDF11- or vehicle-treated were incubated on membranes of the phospho-proteomics platforms (human Phospho-MAPK, 23 different MAPKs and other serine/threonine kinases), as described in "Materials and Methods". (b). Human Phospho-MAPK array coordinates. (c). The graph shows the relative fold change of proteins with significant difference upon GDF11 treatment, setting 1 for control. Protein levels with higher than ± 1.5 folds indicated by dotted lines are considered as the differentially expressed proteins.

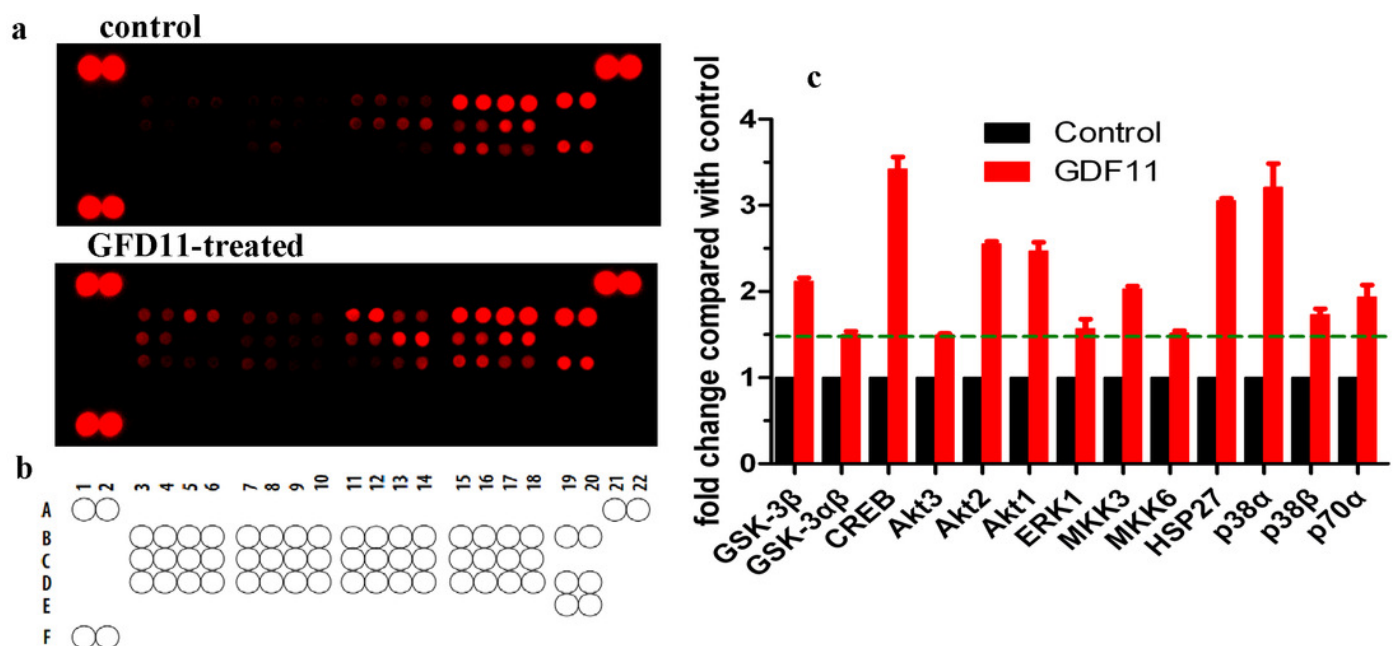
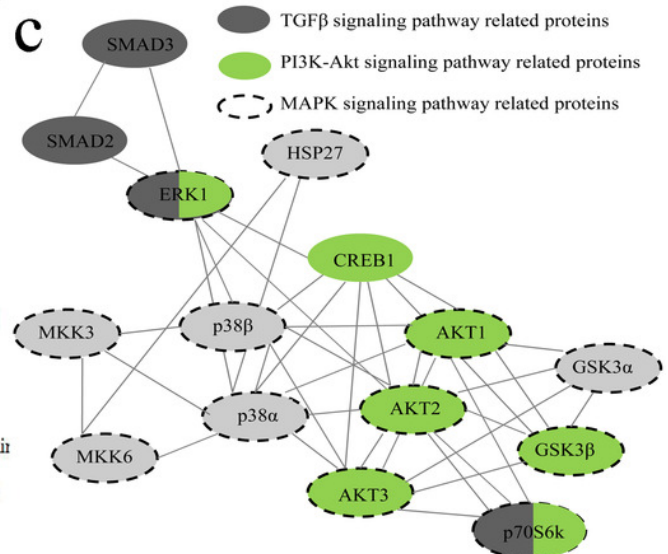
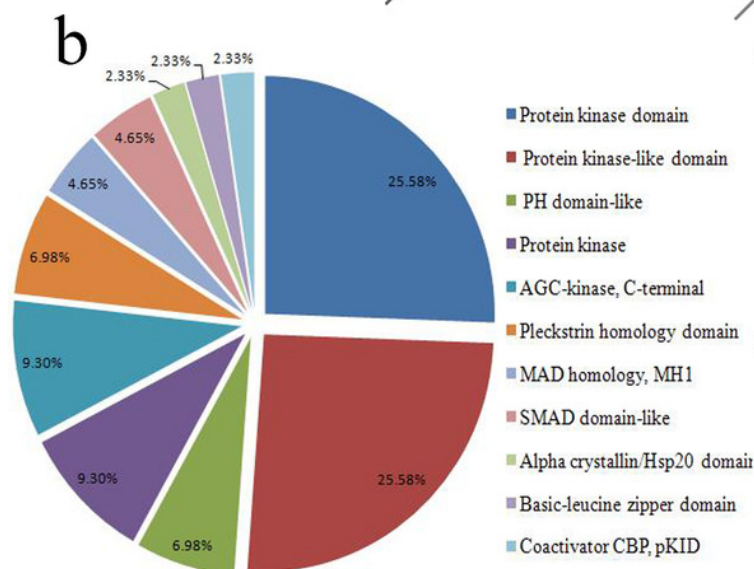
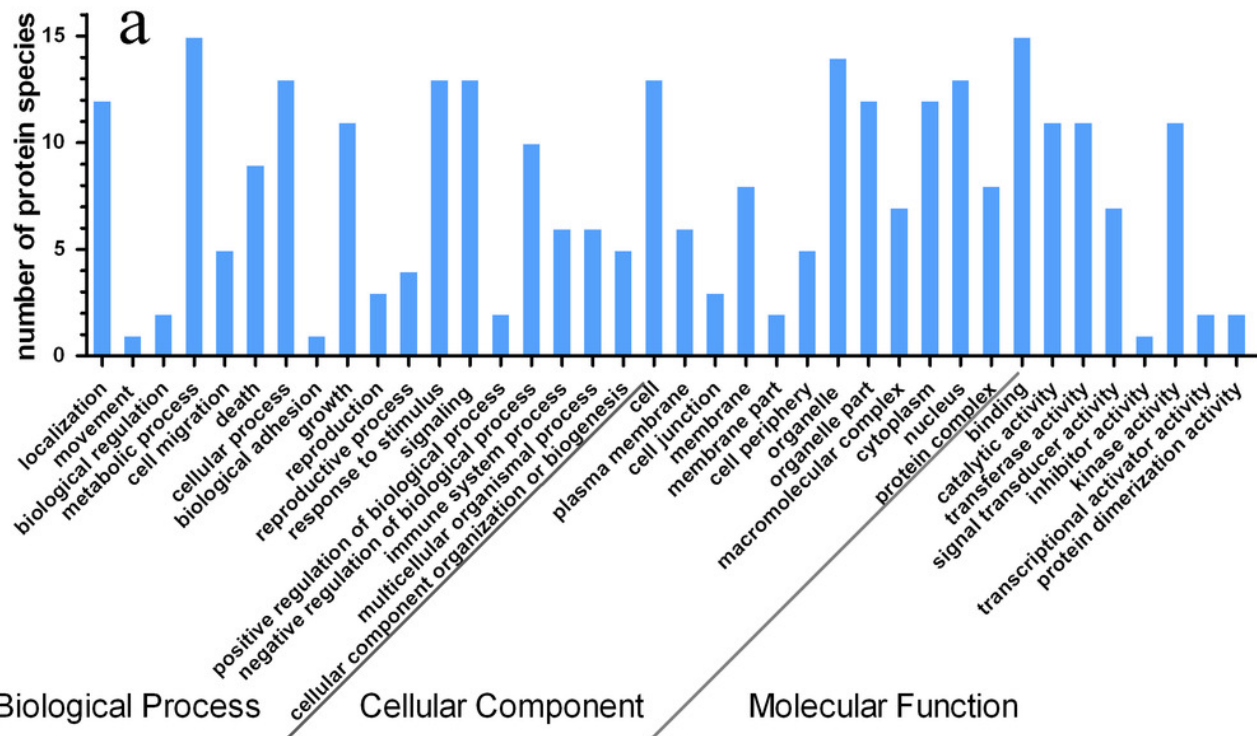



Figure 6

Functional classification and protein-protein interaction of the differentially expressed proteins in GDF11-treated C17.2 cells and control.

(a). According to GO annotation, the differentially expressed proteins between GDF11-treated cells and control were mainly clustered into 38 functional groups, including 18 biological processes, 12 cellular components, and 8 molecular functions. (b). Protein domain categories of the differentially expressed proteins were annotated by InterProScan. (c). The protein-protein interaction network of the differentially expressed proteins was analyzed by KEGG) database.

The subcellular location of the differentially expressed proteins

Proteins	Subcellular Location	Fold Changes
GSK-3β	cytoplasm	2.12
GSK-3α	nucleus	1.50
CREB	nucleus	3.42
Akt2	cytoplasm	2.55
Akt1	cytoplasm	2.47
ERK1	cytoplasm	1.57
MKK3	nucleus	2.03
HSP27	nucleus	3.05
P38α	cytoplasm	3.21
p38β	cytoplasm, nucleus	1.73
p70s6k	nucleus	1.93
Smad2	mitochondria	1.99
Smad3	cytoplasm, nucleus	2.01
Akt3	cytoplasm	1.50
MKK6	cytoplasm	1.52