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Extracellular point mutations in FGFR2 result in elevated Erk1/2 activation and perturbation of neuronal differentiation

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Short title: Effect of extracellular mutations on FGFR2 signalling

Abbreviations: FGFR2: fibroblast growth factor receptor 2, Erk1/2: extracellular signal regulated kinase 1/2, PI3K: phosphatidylinositol-3-kinase, WT: wild type, S252W: serine252 tryptophan substitution, P253R: proline253 arginine substitution

Synopsis

Two independent, gain-of-function point mutations (Ser252Trp and Pro253Arg) in the extracellular region of the fibroblast growth factor receptor 2 (FGFR2) increase the binding affinity for the growth factor. The effect of this enhanced growth factor binding by these mutants is expected to be an increase in activation of regular signalling pathways from FGFR2 as a result of more receptors being engaged by ligand at any given time. Using PC12 cells as a model cell system we investigated the effect of these mutations on protein phosphorylation including the receptor, the activation of downstream signalling pathways and cell differentiation. Our data show that the effects of both these extracellular mutations have unexpected intracellular phenotypes and cellular responses. Receptor phosphorylation was altered in both the ligand-stimulated and unstimulated states. The mutants also resulted in differential phosphorylation of a number of intracellular proteins. Both mutations resulted in enhanced Erk1/2 activation. Although Erk1/2 activation is believed to transduce signals resulting in cell differentiation, this response was abrogated in the cells expressing the mutant receptors. Our data demonstrate that single extracellular point mutations in the FGFR2 have a profound effect on intracellular signalling and ultimately on cell fate.

Key words: FGFR2, Apert syndrome, Erk1/2, PC12 cells, differentiation, MAP kinase, GFP, green fluorescent protein

Introduction

Fibroblast growth factor receptors (FGFRs) are receptor tyrosine kinases that play important roles in various cellular processes, such as mitogenesis, growth regulation, differentiation and specific developmental processes. FGFRs contain an extracellular ligand-binding region, a single membrane-spanning region and an intracellular region containing the tyrosine kinase domain (reviewed in [1-3]). The fibroblast growth factor receptor family comprises four different genes (*fgfr1-4*), which are alternatively spliced to create numerous isoforms that portray some tissue-specific expression (reviewed in [4]). Upon ligand binding in the presence of auxiliary glycosaminoglycans (such as heparan sulphate), these receptors autophosphorylate intracellular tyrosine residues. This phosphorylation event facilitates the recruitment of numerous signalling proteins [5, 6] which subsequently activate various signalling pathways downstream of the FGFR, including activation of the Erk1/2 pathway.

Previous studies have shown that two extracellular point mutations, S252W and P253R, alter the ligand-binding affinity and selectivity of the receptor *in vitro* [7, 8]. These mutations thus provide a unique model system to investigate the effects of perturbation of growth factor recognition on intracellular phenotype. Since these mutations are known to cause Apert syndrome, revealed by phenotypic craniosynostosis and syndactyly, earlier studies investigating the effects of these gain-of-function mutations have focused on cellular aspects such as behaviour and cell fate of osteoblasts and bone precursor cell lines [9-11]. A more recent report highlighted that the S252W mutation is able to drive positive selection in human spermatogonia [12]. However, few data exist on the effects of

these extracellular mutations on the FGFR2 molecule itself and downstream signal transduction.

We adopted PC12 cells to investigate the effects of the S252W and P253R mutations of FGFR2 on intracellular signalling and determination of cell fate. PC12 cells are neuronal precursor-like cells that respond to prolonged Erk1/2 activation following FGF stimulation with differentiation (i.e. extension of neurites similar to those produced by sympathetic neurons in primary culture) [13, 14]. In addition to having been extensively used to study receptor tyrosine kinase signalling pathways, they also provide an excellent cell system to analyse how changes in signalling from the FGFR2 mutations affect neuronal differentiation. Based on the previously described 'gain-of-function' attribute of the mutant receptors, it might have been expected that PC12 cells expressing these mutant forms of the FGFR2 would demonstrate enhanced responses to FGF stimulation compared to cells expressing the wild type FGFR2. Interestingly, whilst the expression of wild type FGFR2 resulted in PC12 cells differentiation in response to FGF9 stimulation, the expression of the mutant receptors resulted in a reduction of differentiation despite causing the enhanced activation of the Erk1/2 pathway. We thus report that the S252W and P253R mutations in the extracellular region of FGFR2 effect a significant perturbation of downstream signalling from this receptor as well as gross effects on neuronal cell differentiation and cell fate.

Experimental

Materials

FGF9 was from R&D systems and NGF from Sigma. The anti-phospho-Erk1/2 antibody was purchased from Cell Signaling Technology, the anti-Erk1 from Santa Cruz, the anti-GFP-antibody from Rockland and the anti-Grb2 antibody from BD Biosciences. Secondary horseradish peroxidase-conjugated secondary antibodies were obtained from Sigma. The biotinylation kit for cell surface protein isolation was purchased from Pierce.

Constructs

The cDNAs encoding FGFR2(IIIc) and FGFR2(IIIc)-S252W were kind gifts from John Heath (University of Birmingham, UK) and were PCR amplified and cloned in frame into the pEGFP vector (Clontech). The P253R mutation was introduced using the Quikchange mutagenesis kit (Stratagene) with appropriate primers encoding the single base pair change according to the manufacturer's instructions.

Cell culture

Parental PC12 cells (ECACC, accession number 88022401) were cultured in DMEM supplemented with 5% foetal bovine serum and 10% horse serum. Prior to stimulation cells were serum-starved for 18 hours in DMEM with 0.1% horse serum. Cells were individually transfected with the three receptor constructs by electroporation (300V, 960 μ F in PBS in a 4mm electroporation cuvette) and stable cells were selected in 800 μ g/ml G418 for two weeks with regular medium changes. Individual clones were isolated by dilution cloning in 96 well plates and screening for fluorescence.

Membrane fractionation and cell surface biotinylation

Membrane fractionation was carried out as described previously [15] using three 10cm dishes per sample. Biotinylation and isolation of cell surface proteins was carried out using the Pierce Cell Surface Protein Isolation Kit. 1×10^5 cells were treated according to manufacturer's instructions. However, the biotinylation time was increased to 90 minutes and the cell lysate was incubated with the immobilised NeutrAvidin gel for two hours at 4°C.

Stimulation, differentiation and proliferation assays

For western blot analysis, cells were seeded on tissue culture dishes coated with poly-D-lysine and serum-starved overnight. Cells were stimulated with 10ng/ml FGF9 in the presence of 1µg/ml heparan sulphate, lysed in 20mM TrisHCl pH 7.5, 138mM NaCl, 1mM EGTA, 20mM β-glycerophosphate, 10% (w/v) glycerol, 1mM sodium orthovanadate, 20mM sodium fluoride, supplemented with 1% (v/v) protease inhibitor cocktail III (Calbiochem) and subjected to SDS-PAGE and Western blotting with the appropriate antibodies.

For the differentiation assay, 1×10^5 cells were seeded on poly-D-lysine-coated tissue culture dishes. After 18-24 hours, the medium was changed to differentiation medium (0.1% horse serum and 20ng/ml FGF9 and 1µg/ml heparan sulphate or 100ng/ml NGF). At least 500 cells were examined for the presence of neurites at various times as indicated. The differentiation medium was replaced every 24 hours. The extent of FGF9-

induced neurite outgrowth was measured as the percentage of cells with neurites greater than two cell diameters in length. Images of random fields of view were obtained using a digital camera fitted to a Zeiss Axiovert 25 microscope.

The number of viable cells at a given time after treatment was measured using an MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Invitrogen) cell proliferation assay. Cells were seeded in triplicate for each time point as for the differentiation assay. When changing the medium to differentiation medium, cells that remained in suspension were also collected and re-added to the respective well to ensure cell numbers were the same as upon seeding. The medium was not replaced to prevent loss of cells, but FGF9 and NGF were replenished 48 hours after the first addition. MTT at a concentration of 5mg/ml in phenol red-free DMEM was added to the cells at a final concentration of 1mg/ml and incubated for 40 minutes at 37°C. The dye was solubilised using acidified isopropanol and its absorbance measured at 570nm (with background subtraction 650nm).

Confocal microscopy

Cells were seeded on glass coverslips coated with poly-D-lysine and fixed in 4% paraformaldehyde. Coverslips were washed in PBS pH 8.0 and mounted on glass slides on a drop of mounting medium (0.1% anti-fade agent 1,4-phenylenediamine (Fluka) in 50% (v/v) glycerol in PBS pH 7.5-8.0). Slides were analysed using a Leica TCS SP2 system with a 63x oil immersion objective. GFP was excited at 488 nm using an argon visible light laser and its emission was detected using 514/10 nm band selection. Mid-

sections of the cells were imaged to avoid interference from cell attachment to glass. Fluorescence images were collected using a photomultiplier tube interfaced to an Intel Pentium II system running the Leica TCS NT control software.

Stage 2(a) POST-PRINT

Results

Creation of PC12 cells expressing the wild type and mutant FGFR2s

The PC12 cell line was chosen to create a model system in which to assess the effects of the S252W and P253R FGFR2 mutations. PC12 cells (rat pheochromocytoma cells originally derived from the adrenal medulla) respond to transient Erk1/2 activation by proliferation, whereas sustained activation of this kinase results in morphologic and biochemical differentiation into sympathetic neuron-like cells [13, 14]. In the light of previous work reporting a role for FGFR signalling in neuronal fate determination, migration, differentiation and survival [16-18], they provide an excellent model to assess the effects of the two mutations in the extracellular domain of the FGFR2 on signalling from this receptor.

To analyse differences in signal transduction PC12 cell lines stably expressing the wild type (WT), S252W or P253R FGFR2(IIIc) respectively were created. Several clonal cell lines were analysed by western blotting (Figure 1A). Those expressing similar amounts of each receptor (WT-C6, S252W-C2, P253R-C3) were selected for further experiments after determining that cellular responses did not vary between clones (data not shown). Confocal imaging revealed that all three GFP-tagged receptors localised primarily to the plasma membrane. A small amount of receptor was observed in intracellular compartments presumably undergoing biosynthetic processing (Figure 1B).

Despite the total expression levels being similar in all three cell lines (Figure 1A), to confirm that any alterations in signalling from the wild type and mutant receptors were

not due to differential expression at the cell surface we performed sub-cellular fractionation and biotinylation followed by isolation of membrane proteins (Figure 1C and D). Sub-cellular fractionation and biotinylation experiments revealed that all three receptors are located in the membrane, thereby confirming correct posttranslational targeting of the mature receptor. Separation of the receptors expressed at the cell surface from those located within the cell interior using biotinylation revealed that the overall expression of the three different receptors in the plasma membrane was comparable (Figure 1D).

The two mutant FGFR2s are differently phosphorylated

PC12 cells were stimulated with FGF9 to analyse changes in overall cellular tyrosine phosphorylation. FGF9 is specific for FGFR2(IIIc) [19, 20] and does not activate the endogenously expressed FGFR1 [21]. Since PC12 cells do not express FGFR2 [21], the use of FGF9 therefore allows more specific activation of the expressed FGFR-GFP constructs. Previous reports have shown that FGF9 binds to the mutant receptors with greater affinity. In addition it has been reported that FGF9 induces mitogenesis to a greater extent in cells expressing the mutant receptors compared to the wild type FGFR2 [8, 22]. An increase in receptor phosphorylation would generally be assumed to result of the increased affinity since the overall population of receptors which are activated is increased.

Assessment of FGFR2 phosphorylation in the presence and absence of the two mutations revealed that all three variants of the FGFR2 displayed a stimulation-dependent increase

in phosphorylation (Figure 2), confirming that the GFP-tag did not interfere with the kinase activity of the receptor. Interestingly, a straightforward increase in ligand-stimulated receptor phosphorylation that is concomitant with the reported increase in ligand binding was not observed (Figure 2). All three receptors displayed high basal phosphorylation. Interestingly, the level of basal receptor phosphorylation was lower in the presence of the S252W mutation, whereas it was most prominent in the presence of the P253R mutation. This indicates that the mutations have more diverse effects on the FGFR2 than simply resulting in an increased ligand affinity. It was further noticeable, that whereas phosphorylation of the wild type FGFR2 did not further increase upon prolonged exposure of cells to FGF9, both mutant receptors showed increased receptor phosphorylation up to 60 minutes (Figure 2). This observation is commensurate with the increased ligand binding affinity of the mutant receptors. This increased receptor phosphorylation at later time points is likely to affect recruitment of signalling proteins to the FGFR2 and subsequent activation of downstream signalling pathways.

Effects of the mutations on FGFR2 signalling pathways

Several proteins were found to be differentially tyrosine phosphorylated in cells expressing the S252W or P253R receptors (Figure 2, indicated by arrows). Erk1/2 is one of the main downstream targets of activated FGFRs [2], and was found to be one of the proteins differentially phosphorylated in cells expressing either the S252W or the P253R mutant receptors compared to the wild type FGFR2 (Figure 2, phosphoproteins of 42 and 44 kDa as indicated). Erk1/2 activation via the Ras/Raf/MEK pathway occurs after recruitment of the guanine nucleotide exchange factor Sos to the plasma membrane by

associating with the adaptor protein Grb2. Differential phosphorylation of proteins involved in this pathway was additionally observed in addition to the changes in Erk1/2 phosphorylation. These included the major Grb2-recruiting protein downstream of the FGF receptor, FRS2, as well as the adaptor protein Shc (Figure 2). This further supports a significant effect of the two extracellular mutations on one of the main signalling pathways activated by FGF receptor stimulation.

FGF9 stimulation of untransfected PC12 cells did not result in significant Erk1/2 phosphorylation, which indicates that the stimulation of Erk1/2 phosphorylation was not due to activation of endogenous FGFRs and that differences observed are the result of the introduction of the transfected variants of FGFR2 (Figure 3A). Detailed analysis of Erk1/2 phosphorylation following activation of all three receptors revealed striking differences in up-regulation of this kinase (Figure 3B). PC12 cells normally respond to FGF stimulation of endogenous FGFR with sustained Erk1/2 phosphorylation [23]. A similar response was observed on FGF9 stimulation of FGFR2 and mutants thereof, however, the level of Erk1/2 phosphorylation was greatly enhanced in cells expressing the mutants (Figure 3B and C). The P253R mutant showed the highest level of Erk1/2 phosphorylation, which increased with prolonged exposure of cells to FGF9 (Figure 3). This effect mirrored the grossly increased level of P253R receptor phosphorylation (Figure 2B). The S252W receptor also caused enhanced activation of Erk1/2 at all time points observed, which is interesting in light of the much lower basal receptor phosphorylation in cells expressing the S252W receptor compared to the P253R receptor.

Additionally, the onset of Erk1/2 phosphorylation was faster in cells expressing either of the mutant receptors compared to the wild type (Figure 3A: five minutes).

Expression of constitutively active MEK1 or Ras has been shown to be sufficient for induction of neurite outgrowth, and the Erk1/2 pathway is therefore a major determinant of the decision as to whether to undergo proliferation or differentiation in PC12 cells. The PI3K pathway is also activated downstream of the FGFR, however it has been shown to be more important for cell survival than differentiation in PC12 cells [24, 25]. Assessment of the phosphorylation levels of Akt revealed minor differences in the activation of PI3K in cells expressing the wild type FGFR2 or one of the two mutant receptors (Figure 3B). Whereas the levels of Akt phosphorylation seemed relatively unchanged in cells expressing the S252W mutant FGFR2, its phosphorylation was slightly upregulated upon FGF9 stimulation of cells expressing the P253R receptor. This mirrors the changes in FGFR2 phosphorylation in the presence of the two mutations to some extent and further highlights the varied effects that the S252W and P253R mutations have on signalling from the FGFR2.

Decreased neurite outgrowth in response to FGF in the presence of the mutations

It is commonly accepted that the Erk1/2 pathway plays a major role in differentiation of PC12 cells and it has been shown that sustained Erk1/2 activation is required and sufficient for the stimulation of neurite outgrowth [26, 27]. We therefore investigated whether the enhanced Erk1/2 activation in the mutant-expressing cells could lead to increased neurite extension. Strikingly, our data revealed the opposite effect. In cells

expressing the S252W or P253R FGFR2 differentiation was impeded, whilst cells expressing the wild type receptor underwent rapid FGF9-stimulated differentiation which was evident within 24 hours (Figure 4). The cells expressing the P253R receptor were able to induce formation of some neurites, but despite the greatly enhanced Erk1/2 activation cells were not able to reach neurite maturation stages. By 48-72 hours of FGF9 stimulation many cells were in the early stages of differentiation (i.e. flattened, less round and with short projections), but could not progress to form extended neurites even after 120 hours of FGF9 stimulation (Figure 4B). Barely any neurite extension was evident in S252W-FGFR2-expressing cells even after 72 hours of FGF9 stimulation (Figure 4B). Activation of the S252W receptor did not even induce flattening of the cells, which remained round and loosely attached to the surface. The ability of the wild type FGFR2 to induce neurites was comparable to the response of untransfected cells to NGF stimulation. However, the neurites induced by FGF-stimulation were less stable and retracted with prolonged exposure (beginning at 72 hours) while NGF-stimulated outgrowth was sustained up to 120 hours. It is unclear why this occurred, but seems to be in accordance with a previous report indicating decreased stability of FGF-induced compared to NGF-induced neurites [13].

To assess whether instead of initiating neurite outgrowth, cells expressing the mutant receptors were undergoing proliferation, the number of viable cells present was assessed using an MTT cell proliferation assay following stimulation of cells with FGF9 for 24 or 72 hours (Figure 5). Somewhat surprisingly, no difference was observed between any of the cell lines assessed, and all cells showed a similar increase in cell number (which is

indicative of similar levels of proliferation). It is unclear why even those cells that begin to differentiate after 24 hours (i.e. cells expressing the wild type FGFR2 stimulated with FGF9 and untransfected PC12 cells stimulated with NGF) showed similar levels of proliferation. This may be due to the residual horse serum contained in the differentiation medium. More importantly however, cells expressing either the S252W or the P253R FGFR2 did not show increased levels of cell proliferation despite portraying enhanced activation of the Erk1/2 pathway.

Discussion

Mutations in the FGFR, including S252W and P253R in the FGFR2, have been previously described as gain-of-function when acting through various mechanisms such as constitutive activation, illegitimate splicing or, as in the case of our mutations, through increased affinity and altered selectivity in ligand binding [12, 28]. This term implies that the S252W and P253R mutations simply result in enhanced activation of the regular signalling pathways as a result of a greater number of receptors being engaged by ligand at any given time. In contrast, our data show that the presence of the mutations results in a more complex intracellular phenotype than can be described by simple up-regulation of FGFR2 signalling.

Our data suggest that the extracellular point mutations affect receptor phosphorylation in both the unstimulated and the FGF9-stimulated state. Whereas in the basal state the S252W receptor was found to be less phosphorylated than the wild type FGFR2, phosphorylation of the P253R receptor was significantly more pronounced. These observations show that the two mutations not only affect ligand binding extracellularly, but also appear to alter the receptor intracellularly to result in differential tyrosine phosphorylation.

It is also particularly interesting to note that both mutant receptors displayed a further increase in tyrosine phosphorylation after prolonged (60 minute) stimulation compared to the wild type FGFR2 (Figure 2). This indicates a decreased capacity of the mutant receptors to down-regulate downstream signalling and appears to contribute to the

observed increase in Erk1/2 activation. We failed to observe any clear differences in receptor internalisation following FGF9 stimulation in PC12 cells (data not shown), although we have shown that the mutant receptors produce defective trafficking following ligand binding in other cell lines (Z.A., A.C.S., J.E.L. manuscript in submission). The observation that all three receptors remained located at the cell surface membrane even following prolonged FGF9 stimulation in PC12 cells suggests that endocytosis plays no role in the differential signalling responses observed in this cell line.

Although an overall increase in receptor phosphorylation could simply be indicative of an increased number of receptors being activated, the possibility that alterations in the stoichiometry of phosphorylation of individual tyrosine residues do not occur as well can not be discarded. It has recently been shown that FGFR1 phosphorylation occurs in a sequential and highly regulated manner [29]. Changes in the interaction of the FGFR2 with FGF ligand, as is the case in both the S252W and P253R mutants, might affect the sequential order of these phosphorylation reactions, which would then result in changes in protein recruitment and signal activation downstream of the receptor.

In addition to alterations in FGFR2 tyrosine phosphorylation, we have shown that signal transduction from the FGFR2 is affected by the presence of both the S252W and the P253R mutations. If the mutations resulted merely in an increase in activated receptors, then all FGFR2-induced signalling pathways would be expected to be up-regulated. Even though the Erk1/2 pathway was greatly up-regulated in cells expressing either of the mutant receptors, the PI3K pathway remained relatively unaffected by the presence of the

mutations, although slight changes in Akt phosphorylation in the three different cell lines could also be observed. Further, analysis of total tyrosine phosphorylation of proteins revealed that not all proteins were phosphorylated to a greater extent in cells expressing the mutant receptors when compared to the wild type receptor (Figure 2, as indicated by arrows). These observations further support the notion that the effects of these mutations on the receptor and resulting downstream response are more complex than a simple gain-of-function effect as described previously.

The mutant receptor-induced up-regulation of the Erk1/2 pathway was not sufficient to result in differentiation of PC12 cells, further demonstrating that both mutations have profound effects on cells' responses to FGF stimulation. The up-regulation of the Erk1/2 pathway is in agreement with the previously reported increase in mitogenesis in cells expressing the S252W FGFR2 [22] and seems to directly reflect the increased ligand binding affinity of this receptor. However, the fact that activation of this pathway neither led to PC12 cell differentiation nor increased proliferation in cells expressing the S252W or P253R receptors indicates that activation of other pathways downstream of the FGFR2 must render these cells incapable of the expected phenotypic response.

Previous studies have implicated prolonged Erk1/2 activation as sufficient to cause neurite outgrowth in this cell line [26, 30, 31]. Over-expression of, for example, the EGFR and the insulin receptor, which also lead to enhanced and prolonged Erk1/2 activation is able to induce PC12 cell differentiation [32, 33]. Our observation therefore not only indicates that the S252W and P253R mutations must affect other FGFR2

activated signalling pathways that contribute to the observed lack of neurite outgrowth, but also show that the commonly accepted dogma that prolonged Erk1/2 activation is sufficient for differentiation [27] may not be correct. It is likely that more complex control mechanisms are in place to provide the changes that prevent neurite outgrowth despite enhanced levels of Erk1/2 activation. For example we have shown that recruitment of the docking protein FRS2 to FGFR2 is altered in the presence of both S252W and the P253R (Z.A., A.C.S., J.E.L., manuscript in submission) suggesting that the early signalling complex recruited to the receptors are fundamentally affected by the extracellular mutations. Alterations in the recruitment of proteins to the receptor, likely as a result of the altered receptor phosphorylation, would be expected to affect not only one signalling pathway, but the activation and interplay of all FGFR2-activated pathways. Further investigation is required to determine the mechanism by which neurite outgrowth is prevented in response to activation of the two mutant receptors despite the enhanced activation of the Erk1/2 pathway.

In conclusion, we have shown the importance of the recognition of extracellular ligands to the specific signal generation downstream of tyrosine kinase receptors such as the FGFR2. This has a profound effect on maintenance of the integrity of cellular responses to a stimulus which are reflected in the ultimate cellular phenotype such as neuronal differentiation. By perturbing the normal signal generation from the FGFR2, the S252W and P253R mutations result in enhanced Erk1/2 activation. However, changes in signal activation downstream of the FGFR2 were found to prevent differentiation of PC12 cells despite this enhanced Erk1/2 activation.

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Figure Legends

Figure 1: Expression of GFP-tagged wild type or mutant FGFR2 in PC12 cells

(A) Pools of PC12 cells stably expressing the WT, S252W or P253R FGFR2-GFP were subjected to dilution cloning. Several clones were isolated and cell lysates were subjected to SDS-PAGE and immunoblotting with an anti-GFP antibody to pick clones of equal expression of each of the receptors. (B) Chosen PC12 clones (WT C6, SW C2, PR C3) expressing each of the GFP-tagged receptors were seeded onto poly-D-lysine-coated coverslips, fixed in 4% paraformaldehyde and analysed by confocal microscopy. Images are representative of at least ten fields of view for each cell line. (C) Subcellular fractionation of untransfected PC12 cells and cell line clones expressing the three FGFR2-GFP constructs was carried out and the cytosolic, C, and membrane, M, fractions were subjected to SDS-PAGE and immunoblotting with an anti-GFP antibody. (D) Cell surface proteins of untransfected PC12 cells and cell clones expressing each of the FGFR2-GFP constructs were labelled with biotin and isolated using immobilised NeutrAvidin gel. An unlabelled sample of cells expressing the P253R FGFR2 was included as a control of labelling ('No label'). Equal volumes of the biotinylated eluate, B, and the flowthrough, FT, were subjected to SDS-PAGE and immunoblotting with an anti-GFP antibody.

Figure 2: Phosphorylation of GFP-tagged receptors

(A) PC12 cell clones expressing each of the GFP-tagged receptors were seeded on poly-D-lysine-coated tissue-culture plates, serum starved overnight and stimulated with 10ng/ml FGF9 and 1 μ g/ml heparan

sulphate for indicated time periods. Cells were lysed and 200 μ g of the respective whole cell lysates were subjected to SDS-PAGE and immunoblotting with an anti-phosphotyrosine antibody (upper panel). A longer exposure of the lower part of the blot is shown to indicate changes in tyrosine phosphorylation of cellular proteins (middle panel). Unidentified proteins are indicated by an asterisk. The blot was stripped and re-probed with an anti-GFP antibody to indicate location of the FGFR2. Since stripping removed large parts of the highly phosphorylated P253R FGFR2, the blot was re-probed with an anti-Grb2 antibody to account for even loading.

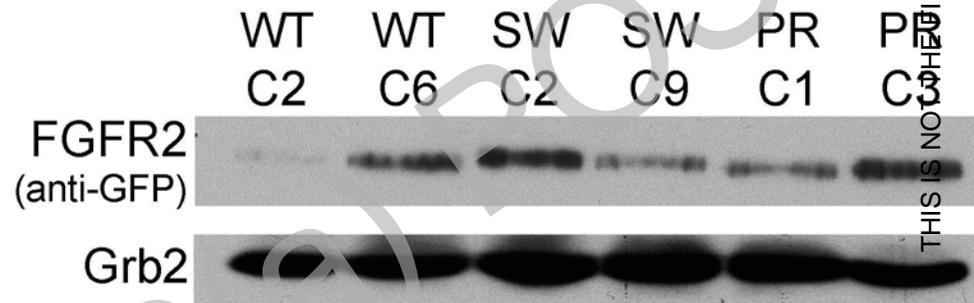
Figure 3: Effect of the S252W and P253R mutations on Erk1/2 activation (A) PC12 cells were stimulated with 10ng/ml FGF9 and 1 μ g/ml heparan sulphate for various time periods following overnight serum-starvation and lysed. 200 μ g of whole cell lysate were subjected to SDS-PAGE and immunoblotting with an anti-phospho-Erk1/2 antibody. The membrane was stripped and re-probed with an anti-Erk1 antibody and an anti-GFP antibody as loading and expression controls respectively. (B) The amount of phospho-Erk1/2 following stimulation of the wild type (solid line), S252W (broken line) or P253R (dotted line) FGFR2 in Figure 3A was quantified using densitometry and the data were represented graphically. The standard error of the mean (SEM) calculated from two independent experiments is indicated by error bars.

Figure 4: Effect of mutations on neurite formation and differentiation Untransfected PC12 cells, or those expressing the wild type, S252W or P253R FGFR2 were seeded on poly-D-lysine-coated plates, allowed to attach for 24 hours with subsequent change to

differentiation medium containing 10ng/ml FGF9 and 1 μ g/ml heparan sulphate or 100ng/ml NGF. (A) Pictures of the cells were taken as indicated. (B) At least 500 cells were scored for neurite outgrowth in each condition. The average data from two independent experiments (one for NGF stimulation) is represented as the percentage of cells that contained neurites more than two cell diameters in length. The standard error of the mean (SEM) is indicated as error bars. WT – solid line, S252W – broken line, P253R – dotted line, PC12 FGF9 – grey triangles, PC12 NGF – circles.

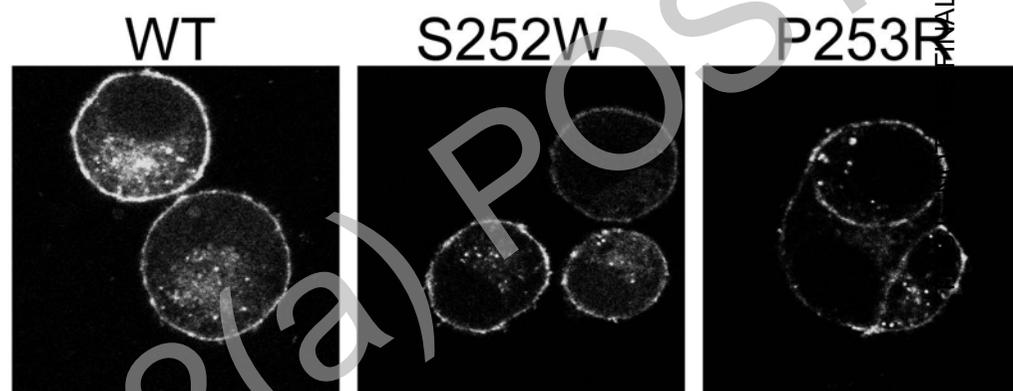
Figure 5: Cells expressing the S252W or P253R FGFR2 do not respond to FGF9 stimulation with increased proliferation rates Untransfected PC12 cells, or those expressing the wild type, S252W or P253R FGFR2 were seeded in triplicate on poly-D-lysine-coated plates, allowed to attach for 24 hours with subsequent change to differentiation medium containing 10ng/ml FGF9 and 1 μ g/ml heparan sulphate or 100ng/ml NGF. The relative number of viable cells was assessed using an MTT cell proliferation assay 0, 24 and 72 hours after the addition of differentiation medium. The data is presented as percentage increase from point '0'. Similar trends were observed in two independent experiments. The standard error of the mean (SEM) is indicated.

Figure 1A



Stage 2 (Pre-proof)

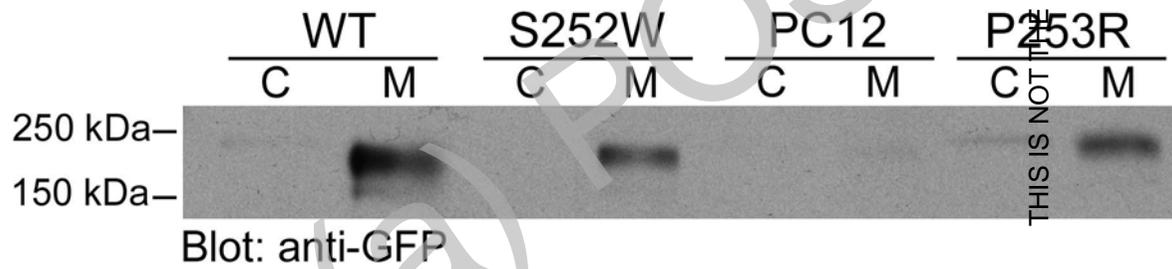
Figure 1B



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Stage 2(a)

Figure 1C



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Stage 2(a) POST-PRINT

Figure 1D

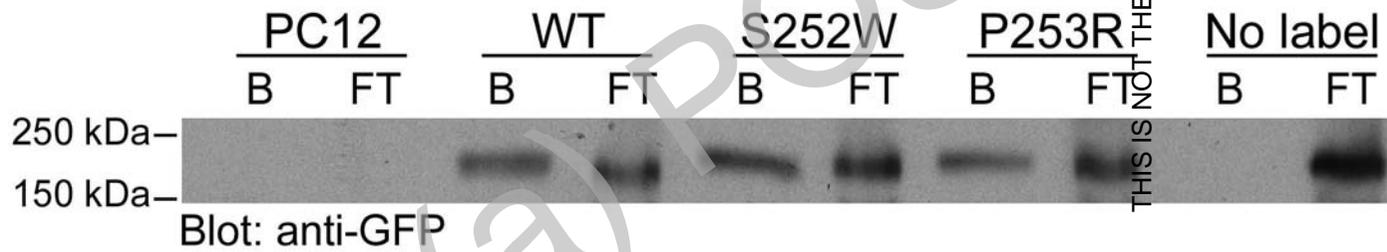
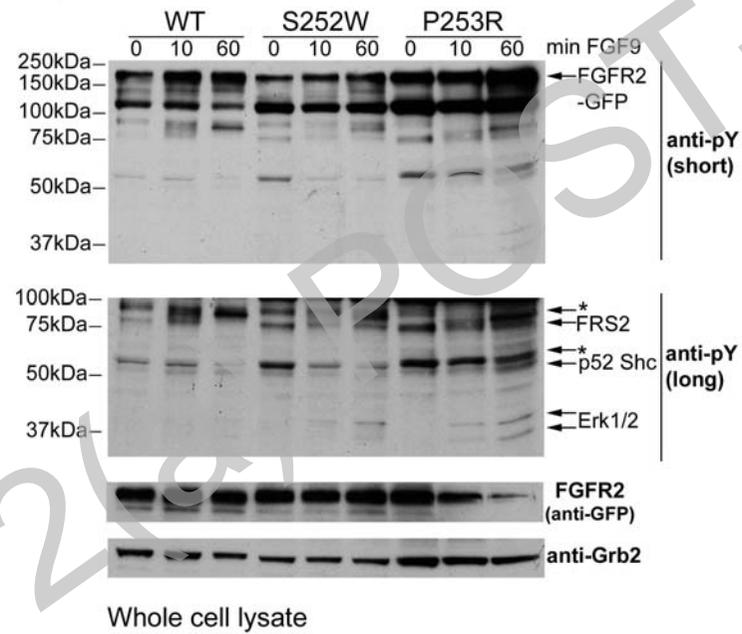


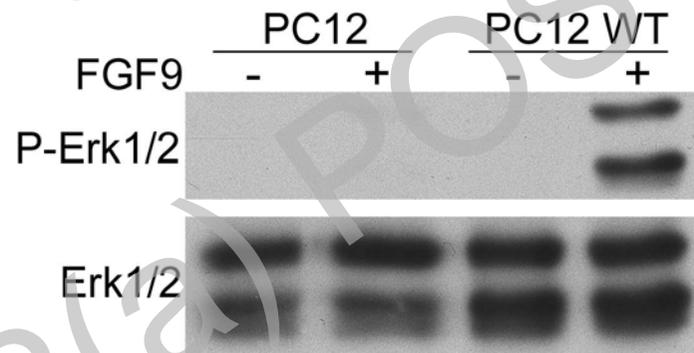
Figure 2



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Stage 2

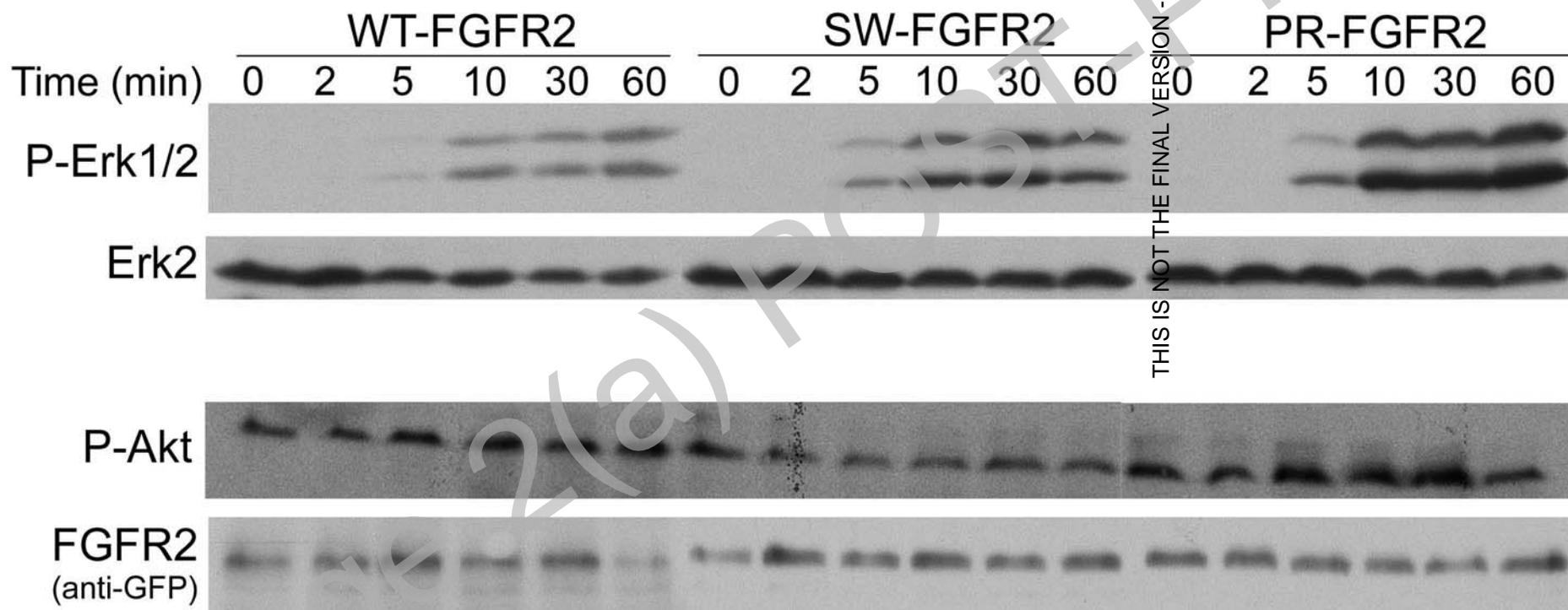
Figure 3A



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Stage 2 (a)

Figure 3B



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Figure 3C

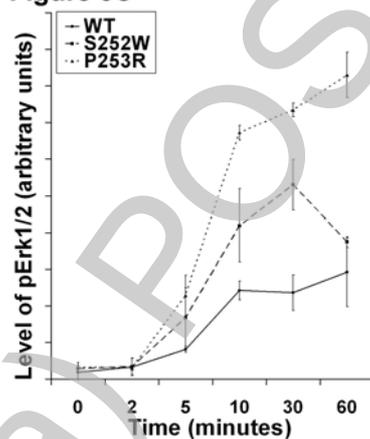
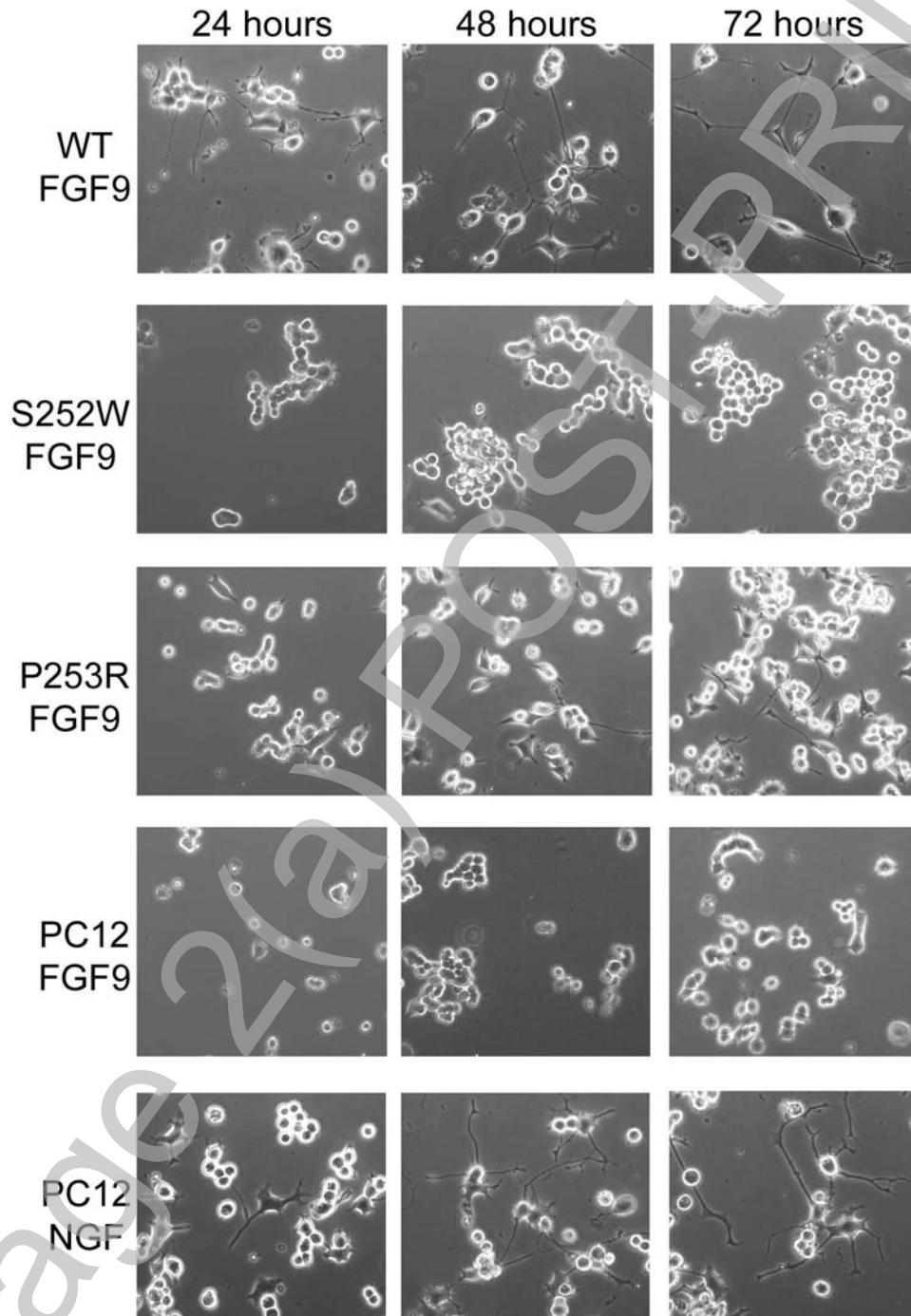
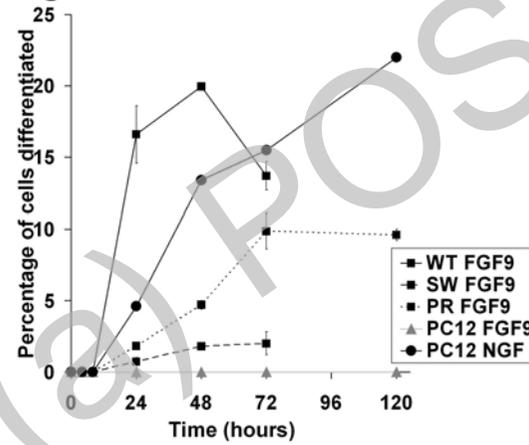


Figure 4A



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Figure 4B



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Figure 5

