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LIM kinases and fetal renal programming.

Keywords

kidney, nephrogenesis, cofilin1, Limk1, Limk2, mitosis, programming.

Abstract

Aims.

Maternal dietary restriction during pregnancy impairs nephron development and results in offspring with fewer nephrons. Cell turnover in the early developing kidney is altered by exposure to maternal dietary restriction and may be regulated by the LIM-kinase family of enzymes. We set out to establish whether disturbance of LIM-kinase activity might play a role in the impairment of nephron formation.

Main Methods.

E12.5 metanephric kidneys and HK2 cells were grown in culture with the pharmacological LIM-kinase inhibitor BMS5. Organs were injected with DiI, imaged and cell numbers measured over 48hrs to assess growth. Cells undergoing mitosis were visualised by pH3 labelling.

Key Findings.

Growth of cultured kidneys reduced to 83% of controls after exposure to BMS5 and final cell number to 25% of control levels after 48hrs. Whilst control and BMS5 treated organs showed cells undergoing mitosis (100±11cells/field vs 113±18cells/field respectively) the proportion in anaphase was considerably diminished with BMS5 treatment (7.8±0.8% vs 0.8±0.6% respectively; $P<0.01$). This was consistent with effects on HK2 cells highlighting a severe impact of BMS5 on formation of the mitotic spindle and centriole positioning. DiI labelled cells migrated in 100% of control cultures vs

0% BMS5 treated organs. The number of nephrogenic precursor cells appeared depleted in whole organs and formation of new nephrons was blocked by exposure to BMS5.

Significance.

Pharmacological blockade of LIM-kinase function in the early developing kidney results in failure of renal development. This is likely due to prevention of dividing cells from completion of mitosis with their resultant loss.

Introduction

Maternal undernutrition during gestation results in developmental changes which predispose individuals to various non-communicable diseases of adulthood including coronary heart disease, hypertension [1, 2] and type 2 Diabetes mellitus [3, 4]. Furthermore, individuals who were growth impaired during fetal life have a 70% greater risk of developing chronic kidney disease [5]. The underlying causes are still to be fully established, but evidence points to developmental changes in organ systems which result in their limited functional capacity at maturity. Kidney development has been shown to be adversely impacted by maternal undernutrition during gestation with the result that offspring possess fewer nephrons than controls [6-11]. A reduced nephron complement has been strongly associated with a predisposition towards hypertension, coronary heart disease and renal disease [12-14] and animal studies have provided experimental support for these observations [6, 15, 16]. Rats supplied a low protein diet during pregnancy have elevated levels of apoptosis early in renal development which appears to play a significant role in the reduction of the final nephron number [9]. It has been speculated that this may lead to a loss of nephron precursors and is a potential mechanism by which nephron complement is diminished.

Numerous studies have examined the process of maternal macronutrient restriction mediated impairment of kidney development, however there remains no clearly defined molecular pathway by which this occurs. We have previously observed differential regulation of the enzyme Cofilin 1 (*Cfl1*; [17]) in early developing kidneys following exposure to a maternal dietary protein restriction. The actin severing activity of Cofilin1 is regulated by the Lin11, Isl-1 and Mec-3 (LIM) domain containing kinases [18-20] and members of these, notably LIMK1, are robustly expressed in the early developing kidney [21]. In addition to their role in regulating Cofilin1 function, it has been shown that the LIM kinases play a critical role in mitosis. Inhibition of these enzymes using siRNA or a pharmacological agent (N-(5-(1-(2,6-dichlorophenyl)-3-(difluoromethyl)-1H-pyrazol-5-yl)thiazol-2- yl)

cyclopropanecarboxamide; BMS5) prevents cells from progressing through anaphase and they subsequently die [22, 23].

Because of the presence of LIMK1 in developing kidneys along with one of its functional targets (Cofilin1), which is repressed by maternal dietary protein restriction, we set out to determine if there is a role for the LIM kinases in mediating kidney development.

Materials and Methods

Organ Culture

All animal work was approved by the University of Nottingham Animal Welfare Ethical Review Board and was carried out in accordance with Home Office Guidance on the operation of the Animals (Scientific Procedures) Act (Great Britain Home Office, 2000). E11.5-E12.5 ICR mouse kidneys were dissected and kidneys were cultured for either 24hr or 48hr in DMEM/F12 (Sigma, Dorset, UK), supplemented with Penicillin (100 units/ml; Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), streptomycin (100 µg/ml; Invitrogen), recombinant human insulin (10 mg/l; Sigma), human transferrin (5.5 mg/l; Sigma), and sodium selenite (5 µg/l; Sigma), and grown on Millicell cell culture plate inserts (pore size 0.4 µm; Millipore, Watford, Hertfordshire, UK) [24, 25]. The media of the experimental group was supplemented with 50µM BMS5 (Synkinase, Pudong, Shanghai, China).

BMS5 has been shown to selectively inhibit both LIMK1 and LIMK2 without cytotoxic effects or any effect on tubulin or Testis-Specific Kinase 1 in interphase cells [26]. Some inhibition of Protein Kinase

AMP-Activated Catalytic Subunit Alpha 1 (PRKAA1) and Fibroblast Growth Factor Receptor 1 (FGFR1) have also been observed [26].

DiI injections

Cultured kidneys were injected at their periphery using a micro injector (FemtoJet, Eppendorf, Stevenage, UK) with a borosilicate glass capillary needle (GC100TF-15 - Harvard Apparatus, Cambridge, UK). Each target site within the kidney was injected with 5 µL DiI - Fluorescent lipophilic membrane dye (1 mg/ml in ethanol, diluted 1:1 in 0.3 M sucrose in Phosphate buffered saline (PBS); C-7001, CellTracker™ CM-Di I, Molecular Probes, Thermo Fisher Scientific). Each kidney was injected at 3 separate sites and grown for a further 24 hrs.

Organ cell count

Kidneys were dissociated in 0.25 % trypsin/EDTA (Sigma) for 8 minutes at 37°C, after which 10% fetal bovine serum (FBS, Sigma) diluted in PBS was added. Dissociated cells were counted using a haemocytometer (Paul Marienfeld GmbH & Co. KG, 97922 Lauda-Königshofen, Germany).

Whole mount immunofluorescence

Kidneys were fixed in 4% paraformaldehyde (PFA, Sigma) in PBS for 20 minutes, washed in PBS, transferred to tubes containing 0.2% triton X100 (Sigma) in PBS and incubated overnight at 4°C on a tube roller to permeabilise the tissue. Kidneys were then washed three times in PBS and incubated with primary antibodies (diluted in PBS) overnight at 4°C on the tube roller. Kidneys were washed in PBS and incubated with secondary antibodies (diluted in PBS) overnight at 4°C on the tube roller. Kidneys were finally washed again in PBS (3 times) and mounted on microscope slides (pre-prepared with a ring of hardened DPX to preserve the 3d structure of the kidney) in Vectashield Hardset (Vector Laboratories, Peterborough, UK). Primary antibodies: Anti-Calbindin-D-28K (1/100, C9848, Sigma-Aldrich), Anti-Six2 (1/100, 11562-1, Proteintech, Manchester, UK), anti-Histone H3 (phospho S10; 1/500, ab5176, AbCam, Cambridge, UK). Secondary antibodies: Anti-Mouse IgG, Texas red

(1/100, T-862, Invitrogen), Anti-Rabbit IgG, FITC (1/20, F0205, Dako, Ely, Cambridgeshire, UK). For whole mount apoptosis assessment, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was used. Kidneys were prepared as described above for whole mount immunofluorescence, after which the reaction was conducted according to the manufacturer's instructions (In Situ Cell Death Detection Kit, Roche Diagnostics, Welwyn Garden City, Hertfordshire, UK).

Cell culture

Human proximal tubule cells (HK2) were grown and maintained in Dulbecco's Modified Eagle's Medium with 4500 mg/L glucose (Sigma), supplemented with penicillin, streptomycin and 10% FBS for 24hr. Media of experimental groups was supplemented with BMS5 at either 3µM, 10µM or 50µM.

Cell immunofluorescence.

Cells were fixed in 4% PFA (in PBS) for 15 minutes, washed three times with PBS, permeabilised by incubation in 0.1% Triton X-100 (in PBS) for 15 minutes, washed three times in PBS and incubated with primary antibodies (diluted in PBS) overnight at 4°C. The following day, cells were washed three times with PBS and incubated with secondary antibodies (diluted in PBS) for 90 minutes at room temperature, washed in PBS and mounted in Vectashield Hardset with DAPI. Primary antibodies: Anti-Histone H3 (phospho S10) (1/500 - ab5176, AbCam), Anti-Pericentrin (1/200, ab4448, AbCam), Anti-Alpha Tubulin (1/100, T9026, Sigma-Aldrich). Secondary antibodies were the same as those used for whole mount immunofluorescence.

Cell count.

Media was removed and 0.25 % trypsin/EDTA was added to the wells for 4 minutes at 37°C after which media containing 10% FBS was added to quench the trypsin activity. Cells were counted using a haemocytometer.

Fluorescence image acquisition.

Cells and organs were imaged using a Leica DM5000 B microscope, Leica DFC420 camera and LAS v.3.8 software (Leica Microsystems (UK) Ltd, Milton Keynes, UK).

Mitosis and anaphase counting protocol

Kidneys and cells were fixed and labelled for pH3. Images were taken (cells: 4 images per coverslip; organs: 5 images per organ) at x20 magnification and the total number of pH3 stained cells were counted in each field of view. We additionally counted the number pH3 labelled nuclei showing a clear separation between chromosomes and assigned these as being in anaphase.

Statistical analyses

All experiments had an n number of at least 3 unless otherwise specified. Data are expressed throughout as mean±SEM. Statistical analyses of cell counts, mitosis counts and growth data were carried out using one-way ANOVA analyses with SPSS v20.0. In the event of a significant ANOVA outcome ($P<0.05$), a Tukey's post-hoc test was performed.

Results

Inhibition of LIM kinase activity severely impairs kidney growth in culture and results in cell deletion.

The surface area of fetal kidneys grown in culture increased by 130±2% after 24hrs (n=11) and 151±4% after 48hrs (n=6). Treatment with the LIM kinase inhibitor, BMS5, in the culture media (50μM) slightly, but significantly slowed organ expansion to 121±3% after 24hrs (n=11; P=0.032 c.f. control) and 125±4% after 48hrs (n=6; P<0.001 c.f. control). Control organs showed a clear ureteric tree and dense regions of cells around the UB tips under light microscopy (Figure 1 A, C & E – asterisks), but, despite the seemingly small effect on surface area increase with BMS5, structurally these organs were greatly affected with the dense regions of mesenchymal cells surrounding UB tips being lost after application of BMS5 (Figure 1 B, D & F – arrowheads). Kidneys exposed to LIM kinase inhibitor appeared to stop developing altogether with no further branching of the ureteric tree observed during the culture period (Figure 1 B, D & F - arrows).

To further assess growth, we micro-injected a subset of organs at their periphery with the fluorescent lipophilic dye, DiI. Movement of cells labelled with DiI (Figure 1 G & H) outwards from the initial injection site (Figure 1H arrow) was seen in all control organs over 24 hours in culture leading to separation of DiI labelled spots in 6 out of 7 control organs examined. Neither movement nor separation of DiI spots occurred in any of the BMS5 treated organs (Figure 1I & Figure 1J arrow).

Cell number in cultured whole fetal kidneys was also considerably affected by incubation with BMS5. Control organs comprised 144,375±31,291 cells after 48hrs of culture, whilst those exposed to BMS5 had only 35,833±12,114 cells (25% of control value; P=0.032). We observed widespread apoptosis after 18 hours of exposure to BMS5.

Inhibition of LIM kinase prevents progression through mitosis in HK2 cells and developing kidneys.

The influence of LIM kinase inhibition on cell cycle progression has been shown in a variety of cell types, but we wished to confirm this in cells from a renal lineage. Therefore, we examined the effect of exposure to the LIM kinase inhibitor on HK2 cells. In line with previous reports we observed impaired mitotic spindle positioning in response to higher concentrations of BMS5 treatment. Spindles were correctly positioned in control cells but were frequently absent or mis-localised in dividing cells exposed to BMS5 (Figure 2A). We noted that in pH3 positive cells cultured in BMS5 at 50μM, α-tubulin was exclusively localised to a few isolated focal asters of microtubules within the nucleus (Figure 2A - arrow).

HK2 cells grown in the presence of BMS5 also showed an increase in the number, and aberrant positioning, of centrioles. Pericentrin labelling demonstrated that inhibition of LIM kinase activity led to over proliferation and mis-localisation of centrosomes (i.e. Pericentrin not co-localised with α-tubulin foci) during mitosis (Figure 2B), but had no effect on these structures in interphase cells (Figure 2C).

Inhibition of LIM kinase activity in HK2 cells resulted in cells becoming stalled in metaphase with significantly fewer cells progressing to anaphase than controls. Quantification of cells labelled for pH3 showed that in control cultures, 13.6%±2.1% of pH3 labelled cells were in anaphase, whilst only 1.4%±1.4% (P=0.002) of cells treated with 10μM BMS5 and 0% of cells treated with 50μM BMS5 reached anaphase (Figure 3A; P=0.001). The disruption of mitosis coincided with a considerable depletion of dividing HK2 cells in culture after exposure to BMS5. Control populations had 36094±2479 cells after 24hrs growth, whilst those incubated with BMS5 showed a dose dependent reduction in cell number (3μM -> 24,844±3,474, 10μM -> 17,969±1,700 and 50μM -> 5,938±541;

P<0.001). This was not seen for confluent cells where numbers were consistent across treatments (0µM -> 540,625±9,544 and 50µM -> 528,281±9,817; P=0.123).

Since BMS5 induced stalling of mitosis in HK2 cells, we examined the frequency of anaphase nuclei in metanephric organ cultures using phospho-histone H3 (pH3) labelling. The number of cells undergoing mitosis, measured by counting nuclei positive for pH3, was similar in all groups (Figure 3Bi). However, detailed analysis of mitotic figures with DAPI staining showed that 7.8%±0.82 of pH3 labelled nuclei were in anaphase in controls cells while organs cultured in 50µM BMS5 had only 0.79%±0.57 (P<0.01) of pH3 labelled nuclei in anaphase (Figure 3Bii).

Because the ureteric tree appeared to have stopped growing in organs treated with LIM kinase inhibitor we wished to identify which particular cell populations were affected by this treatment. To determine this we immunolabelled whole-mount organs with a calbindin antibody to identify the ureteric tree and with a Six2 antibody to label nephron progenitor cells. After 24hrs exposure to BMS5, the calbindin labelled cells of the ureteric tree remained intact, but Six2 expression was considerably downregulated (Figure 3C). After 48hrs exposure, organs were so badly affected that, although no Six2 expressing cells could be seen the tissues were too disrupted to enable adequate images to be generated. Other cell types were not assessed in these experiments.

Discussion

Fetal programming of nephron number [6, 10, 11, 27] has been shown to be, in part, mediated by an alteration in cell turnover in the early developing kidney [9]. Inappropriate induction of apoptosis could deplete populations of cells, reducing the numbers available for nephrogenesis. Previous studies showed that maternal dietary restriction resulted in a reduced final nephron number and that this differed depending upon the stage of pregnancy when restriction was imposed [2, 17]. This may be attributable in part to loss of cells by inappropriate apoptosis during the early stages of development [9]. Several genes (including the *Cofilin1* transcript – *Cfl1*) are downregulated following maternal undernutrition [17]. Cofilin1 protein is expressed in cap mesenchyme, which gives rise to new nephrons – [28-30], ureteric tree [17] and newly induced tubules. Cofilin1 also plays a critical role in both cell migration [31, 32] and proliferation [33]. The partially overlapping expression of LIMK1 and Cofilin1 in some regions of the developing kidney, along with the well characterised ability of LIM kinase to regulate Cofilin function suggested an important role for these proteins during kidney development [21]. The most robust expression of LIMK1 is seen in cells induced to become nephrons (renal vesicles and comma/s-shaped bodies) with much lower expression in the undifferentiated nephrogenic cap mesenchyme. Therefore we wished to explore the possibility that inhibition of LIM kinase activity could have a significant impact on the further development of the induced mesenchyme. It was clear from our observations that following LIM kinase inhibition there was no expansion of pre-existing tubular structures that no new tubules were formed and after 24hrs, much of the organ was disrupted.

In this study we show that pharmacological inhibition of the LIM kinase family of enzymes results in the cessation of renal growth in organ culture, followed by tissue disruption via cell loss. The fetal kidneys grown in this study (E11.5-E12.5) are fairly rudimentary comprising a branching ureteric tree, uninduced cap mesenchymal cells at the ureteric bud tips, stromal cells and some early induced

nephron epithelia [34]. Branching of the ureteric tree stopped, as did the generation of new nephrons. Co-incident with this was failure of anaphase progression and induction of apoptosis. In a renal derived cell line, we confirmed that LIM kinase inhibition disrupted mitotic spindle formation and that dividing cells became rapidly depleted in the presence of the pharmacological inhibitor, BMS5. The mitotic spindles in BMS5 treated cells showed a profusion of astral microtubules emanating from focal points of alpha-tubulin and co-incident with this, multiple unfocussed centrosomes (Figure 2 A &B). These specific structural abnormalities have been observed in HeLa cells depleted of LIMK1, LIMK2 or both using siRNA [22]. Knockdown of LIMK1 was predominantly responsible for the generation of multiple unfocussed centrosomes, whilst targeting of LIMK2 alone mostly impacted upon the generation of large numbers of astral microtubules. Loss of both LIMK1 and LIMK2 generated the most severe phenotype which was identical to that seen with BMS5 treatment, indicating that the LIM kinase activity itself is necessary to prevent these abnormalities from occurring. Our data further suggest that the activity of the LIM kinase family of enzymes may be required for renal development and that their impairment may negatively impact nephron formation due to disruption of mitosis within developing tissues.

One possible explanation for these results is that LIM kinase activity is required at the point of nephron induction to permit continued survival of these cells and that its absence results in a knock on effect for the remainder of the organ, producing the large scale effects on development we observe. In other instances where there is a failure of nephron induction, such as with deletion of Wnt9b [30] or Wnt4 [35], severe renal hypoplasia occurs but there remains a residual rudimentary non-functional kidney. In addition, the loss of FGF8, which is expressed in the renal vesicle and is responsible for the induction of Wnt4 and Lhx1 (itself critical for the further development of the induced tubule [36]), induces mesenchymal cell loss through apoptosis. Either global depletion of

FGF8 [37] or specific metanephric mesenchymal deletion of FGF8 [38] results in organs which are greatly growth impaired with failure of development of nephron epithelia and which show considerable apoptosis of nephron progenitor cells. This would suggest that impairment of nephron formation as a result of LIM kinase inhibition could contribute to the disruption we observed, with evidence from cell culture experiments supporting the idea that failure of mitosis and induction of apoptosis is the most likely mediator.

It was interesting to note that cells expressing Six2 (marking the nephrogenic lineage; [29]) appear to be very sensitive to LIM kinase inhibition as they were almost completely depleted by 24hrs of exposure, unlike the calbindin expressing tubule cells. It is unclear if the nephrogenic cells were actually lost, or whether they switched off expression of Six2. Because there was so little Six2 expression left after BMS5 treatment it was not possible to co-label with apoptotic markers to establish if this cell population was specifically affected by LIM kinase inhibition. Judging from the state of the organs after exposure, it is likely that these cells are lost and this is certainly supported from the cell culture experiments. However, further work will be required to establish if this is the case in vivo.

The consequences of LIM kinase inhibition on kidney formation were far more severe than those seen under conditions of diet induced impairment, with large scale cell death and co-incident prevention of proliferating cells from passing through the final stage of mitosis. Work has shown that cell cycle progression may be altered as a result of maternal dietary restriction [39], but there is no evidence to support the idea that it is completely stalled. It may be the case that the high concentration of BMS5 used in this study resulted in some degree of toxicity, thereby masking the true effect of LIM kinase inhibition alone. However, while this is clearly a possibility the lack of apparent toxicity of this compound at the same high concentration in confluent cultured cells, at

least in terms of cell numbers, would suggest that this dose was reasonably well tolerated and support the hypothesis that the primary effects of LIM kinase inhibition are seen in dividing cells. To fully elucidate the role of the LIM kinases within the developing kidney and their relevance to fetal programming, it will be necessary to examine the consequences of the individual deletion within specific subsets of cells.

We used DiI injections to expand our growth assessment beyond surface area and cell number measurements. The observation of DiI label movement indicated outward growth of the organ and separation of a single site of injection into distinct spots suggested that cells originally labelled in one position had moved independently of the remainder of their group. Cells of the cap mesenchyme have been shown to “swarm” around the ureteric tips remaining in close proximity as growth of the ureteric tree moves those tips outwards [40]. Their motion involves apparently random movements either along, away from or towards their ureteric tip of origin (and even swapping between adjacent cap mesenchyme populations), but they ultimately maintain proximity to the ureteric tips. In so doing, populations of cap mesenchymal cells are able to migrate with the tips. The separation of DiI spots that we observed may highlight these groups of cells which migrate with the ureteric tip growth away from the initial point of injection (Figure 1G & H), although they were not specifically labelled with cap mesenchyme markers in these experiments. We noted that inhibition of LIM kinase activity ceased all outward movement of DiI spots and we never observed any separation of spots in these cultures. Whilst this was probably a consequence of a failure of further ureteric outgrowth, thereby preventing cellular displacement, it may also have been due to inhibition of the cap mesenchymal cells’ ability to migrate. Cofilin1 is required to enable migration in numerous cell types by recycling of actin from the minus end of F-actin filaments, enabling their inclusion at the plus-end in the growth front of a lamellipodium [41] and its activity is regulated by the activity of the LIM kinases [18-20, 42, 43]. This raises the interesting possibility that migration of mesenchymal cells

around the tips of the ureteric tree may be required to permit its continuing outgrowth. Migration of epithelial cells within the ureteric tree has been previously shown to be important for ureteric growth and to be dependent upon the activity of Cofilin-1 and Destrin [44]. It may be the case that the cessation of further ureteric growth in our system was due to the inhibition of this migration or be exclusively a consequence of the failed mitosis we have described, but future work should establish the relative importance of migration of mesenchymal cells. It would also be of interest to determine whether the swarming behaviour of the cap mesenchymal cells is influenced by maternal dietary restriction.

Conclusions

In summary, we have shown that pharmacological inhibition of LIM kinase activity in the early developing kidney has rapid and catastrophic consequences for growth and further development and that this is likely to be mediated by failure of mitosis resulting in cell depletion. These data suggest that LIM kinase function is required during kidney development to permit cell-cycle progression in particular sub-groups of cells and that this is necessary for development of the organ as a whole. Although the effects we report in organ culture are much more severe than those seen following dietary restriction *in utero* they are consistent with the changes in Cofilin1 expression and cell cycle dynamics observed *in vivo*. However, confirmation that changes in LIM kinase activity regulate fetal programming, will require assessment of the activity of these enzymes in specific cell populations within developing kidneys by specifically targeting loss- or gain-of-function mutations to defined cell types during development.

Figure Legends

Figure 1 – (A-F) Effect of BMS5 on metanephric organ culture. E12.5 kidneys cultured ± 50μM BMS5 for 48 hours, arrows indicating loss of further ureteric bud branching, arrow heads indicating loss of definition of mesenchyme. (G-J) E12.5 kidneys grown for 24 hrs after being injected with DiI either without (G & H) or with (I & J) 50μM BMS5. (H) Arrow indicates a DiI injection which has separated into two after 24 hrs growth. (J) Arrow indicates no separation occurs in an example DiI injection site when organs are exposed to BMS5.

Figure 2 – Effects of BMS5 α-tubulin and pericentrin in HK2 cells during mitosis and interphase. (A) HK2 cells labelled for α-tubulin (red) and pH3 (green) during mitosis with DAPI (blue) labelling nuclei. (B) HK2 cells labelled for α-tubulin (red) and pericentrin (green) during mitosis with DAPI (blue) labelling nuclei. (C) HK2 cells labelled for α-tubulin (red) and pericentrin (green) during interphase with DAPI (blue) labelling nuclei.

Figure 3 – Effects of BMS5 on mitosis and Six2 expression in organ culture. E12.5 kidneys grown for 24 hours in 50μM BMS5 were fixed and labelled for pH3. The number of mitotic and anaphase staged nuclei were counted. (A) i. Total number of nuclei in mitosis in controls and BMS5 treated HK2 cells. (ii) Percentage of mitotic nuclei in anaphase in controls and BMS5 treated HK2 cells. (B) i. Total number of nuclei in mitosis in controls and BMS5 treated organs. (ii) Percentage of mitotic nuclei in anaphase in controls and BMS5 treated organs. (C) E12.5 kidneys were grown in 50μM BMS5 for 24 hours, fixed and immune-fluorescently labelled for Six2 (green) and Calbindin (red).

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Figure 1

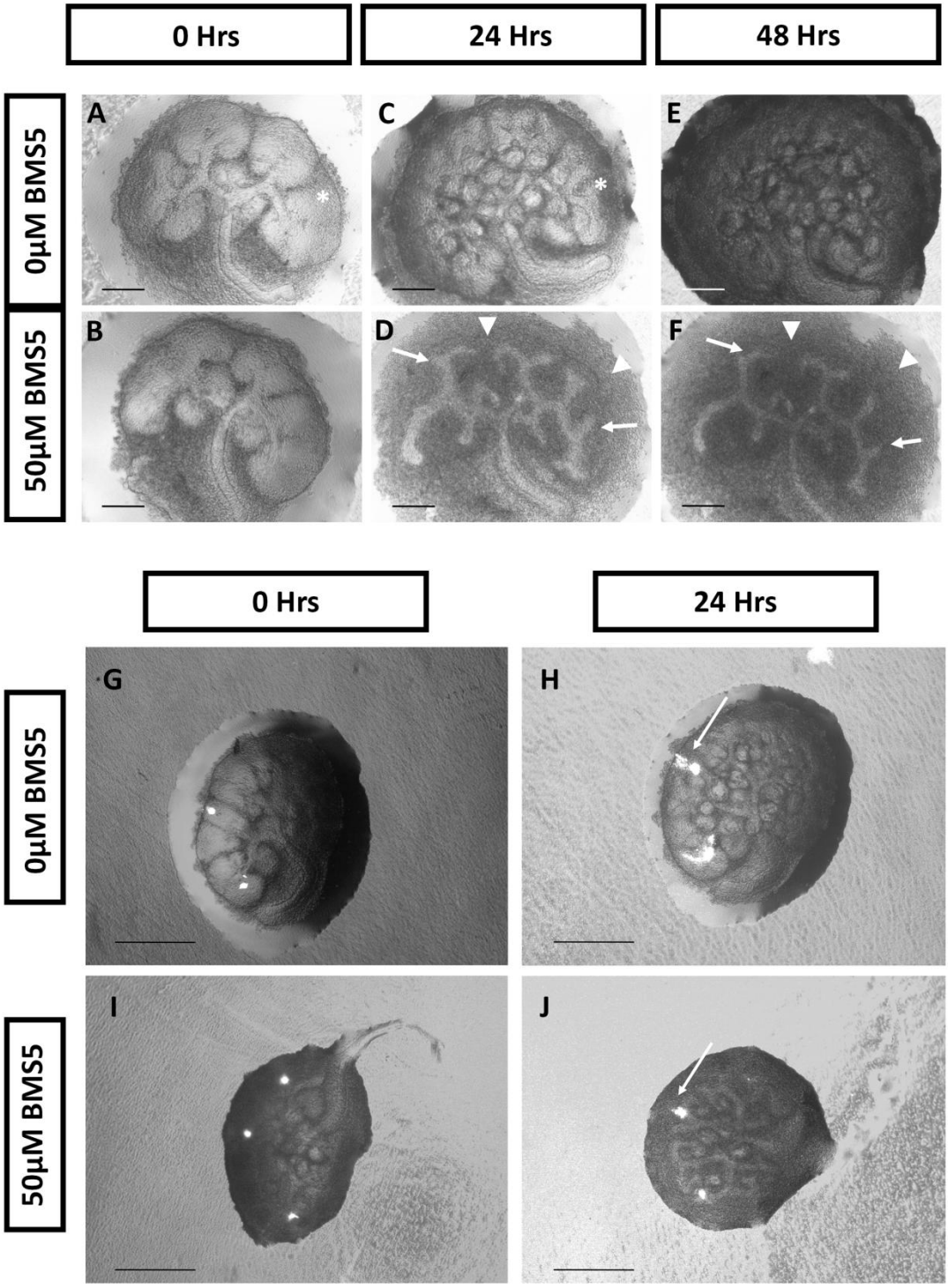

Fig. 1

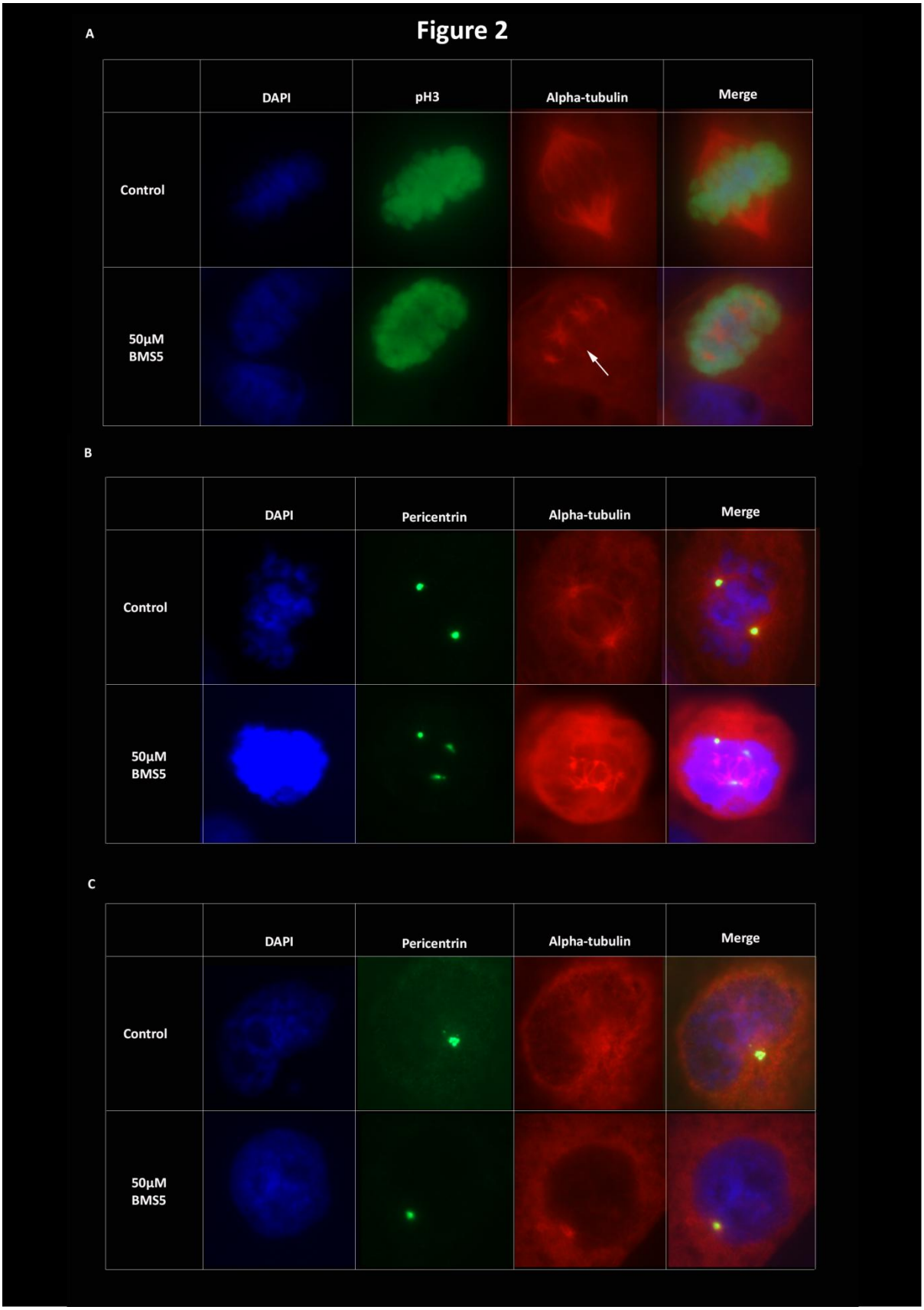

Fig. 2

Figure 3

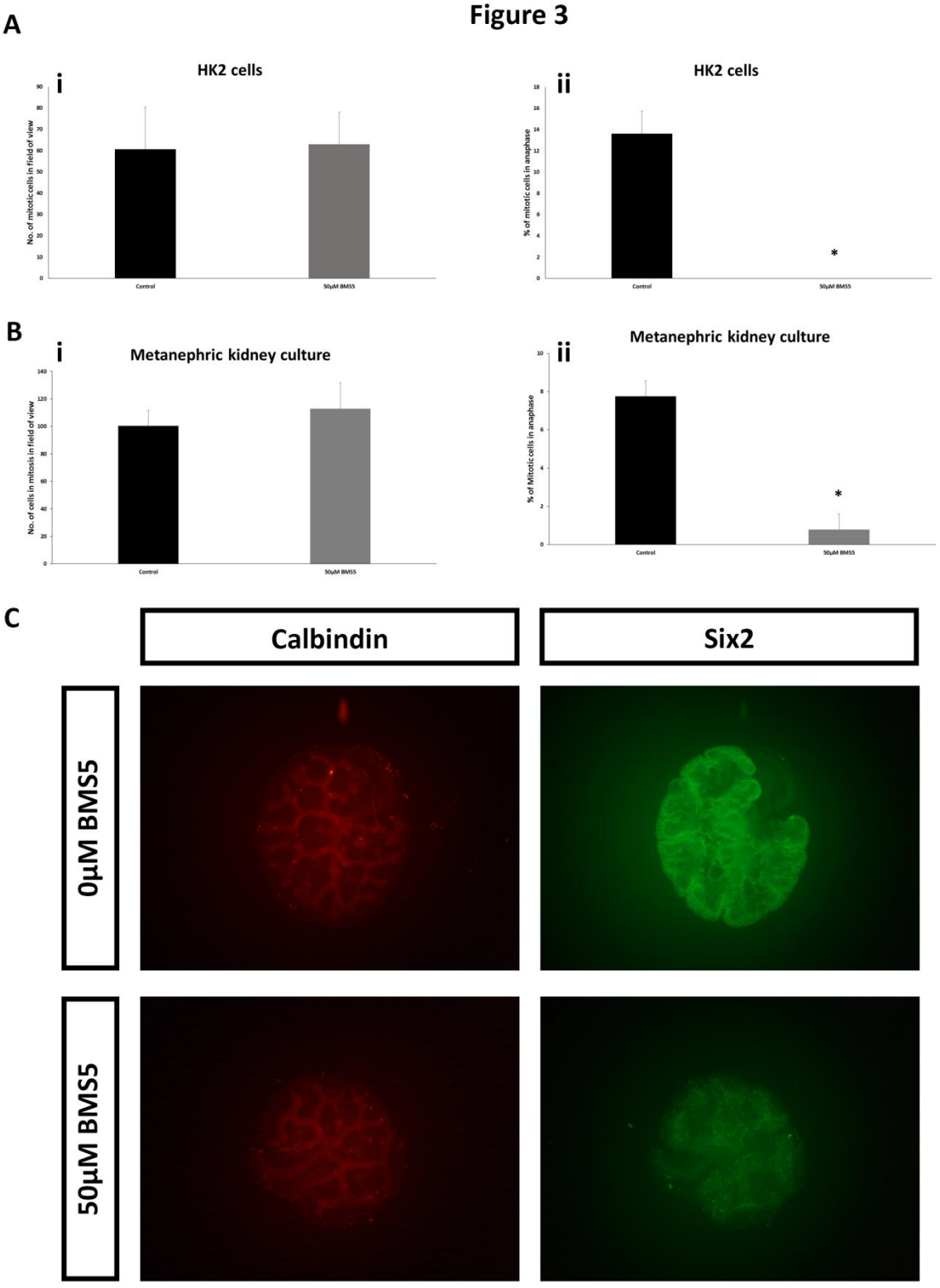

Fig. 3