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ORIGINAL ARTICLE

Exploitation of chick embryo environments to reprogram *MYCN*-amplified neuroblastoma cells to a benign phenotype, lacking detectable MYCN expression

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Neuroblastoma is a paediatric cancer that arises from the sympathetic ganglia (SG) or adrenal gland. Tumours that occur in patients under 18 months of age have a particularly good prognosis and frequently undergo spontaneous regression. This led to the hypothesis that developmental cues in the youngest patients may prompt belated differentiation and/or apoptosis of the tumour cells. To test our hypothesis, we have injected *MYCN*-amplified neuroblastoma cells into the extra embryonic veins of chick embryos at embryonic day 3 (E3) and E6 and analysed the response of these Kelly cells at E10 and E14. Amplification of the *MYCN* gene occurs in up to 30% of tumours and is normally associated with a very poor prognosis. Kelly cells injected at E3 follow neural crest pathways and integrate into neural locations such as SG and the enteric nervous system although never into the adrenal gland. Additionally they migrate to non-neural locations such as the heart, meninges, jaw regions and tail. The cells respond to their respective microenvironments and in SG, some cells differentiate, they show reduced cell division and crucially all cells have undetectable MYCN expression by E10. In non-neural locations, cells form more rapidly dividing clumps and continue to express MYCN. The downregulation of MYCN is dependent on continuous and direct interaction with the sympathetic ganglion environment. We propose that the *MYCN*-amplicon in the Kelly cells retains the ability to correctly interpret the environmental cues leading to downregulation of MYCN.

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INTRODUCTION

Neuroblastoma is an aggressive malignant paediatric tumour that arises from cells of the sympathoadrenal lineage during normal development of the foetus or child and still has a poor outcome for many children.[1–3] Interestingly, neuroblastoma has unusually high levels of spontaneous regression, and this occurs predominantly in younger patients diagnosed before 18 months of age.[4,5] Strikingly, children with extensive metastatic disease—4S stage-may experience complete cure and regression of their disease with only supportive treatment.[4] This leads to a compelling hypothesis that in these youngest patients, developmental cues may prompt the neuroblastoma cells to undergo more favourable differentiation or apoptosis, a phenomenon that has been observed.[6] These findings led us to speculate that the reintroduction of neuroblastoma cells into an appropriate embryonic environment may trigger the cells to differentiate or apoptose rather than form tumours as might be expected in an inappropriate embryonic environment. This was supported by key experiments using malignant melanoma C8161 cells in which these cells introduced into the chick embryo neural tube migrated with the host neural crest and began to express melanocyte or neuronal markers.[7,8] In contrast, cells introduced into an inappropriate embryonic environment, namely the chick eye cup, appeared to form tumours.[9]

In this paper, we have introduced *MYCN* (neuroblastoma-derived v-myc avian myelocytomatosis viral related oncogene)-amplified Kelly cells into the extra embryonic blood vessels of embryonic day 3 (E3) and E6 chick embryos. *MYCN*-amplified tumours normally co-express TrkB and brain-derived neurotrophic factor (BDNF) and generally have the very worst prognosis.[10,11] MYCN is central to a large number of cellular activities including cell cycle progression, apoptosis, senescence and differentiation, although its precise role varies according to circumstance. It requires a partner protein Max and the MYCN:Max heterodimer binds competitively to E boxes in a large number of genes in order to enhance their transcription.[12] In contrast, MYCN:Miz1 acts to suppress expression of target genes. MYCN target genes generally contribute to the aggressive growth of tumours. Targets include telomerase, a negative regulator of p53 (MDM2) and Bmi1, an oncogene that encodes a polycomb transcription factor.[13–15] MYCN also suppresses expression of TrkA, the high affinity nerve growth factor receptor, and p75, the low affinity neurotrophin receptor; both of which are markers of tumours with a good prognosis and in the 4 S stage (the stage that spontaneously regresses).[16]

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MYCN is normally expressed in neural crest cells, is turned off first in cells following the glial cell lineage and later in cells following the neuronal pathway.[17] It has been downregulated in all cells in the chick embryo nervous system by E14. Thus signalling within the neural crest pathway and/or the sympathetic ganglia (SG) appears to downregulate MYCN in normal neural crest cells. However, in *MYCN*-amplified neuroblastoma tumours, it is unclear whether the amplicon, which is of variable size[18] will typically contain the required negative transcriptional controls.[19]

We show in this paper that *MYCN*-amplified Kelly cells introduced into E3 chick embryos target neural crest-derived structures, and MYCN is downregulated in those cells that integrate into and remain in contact with the SG and other neural environments. Cells in an inappropriate embryonic environment retain MYCN expression and their ability to rapidly proliferate. The preferential localisation to neural crest-derived structures and the subsequent loss of MYCN expression also depend critically on the stage of embryonic development as later introduction of Kelly cells at E6 yields very different results.

RESULTS

Kelly cells injected intravenously at E3 and E6 follow different pathways

In initial experiments, we attempted to use the approaches described for malignant melanoma; however, only a small number of cells placed in the neural tube migrated along neural crest pathways and few cells survived and proliferated in the eye cup.[8,9] As neither published method of introducing cells to chick embryos seemed satisfactory for the neuroblastoma cells, we tested whether it would be possible to inject cells into the extra embryonic blood vessels and allow the cells to find their own preferred environment in which to survive and thrive (Figure 1). To enable us to follow the fate of the cells for several days, green fluorescent protein (GFP)-expressing Kelly and BE2C cell lines were prepared. Approximately 200 000 cells were injected into a single embryo enabling us to simultaneously analyse the fate of the cells in many tissues and organs. Surprisingly, both neuroblastoma cell lines largely followed neural crest migration pathways despite their ectopic starting point (Figure 2). In the embryos dissected at E10, cells were routinely found integrated into the SG and within the enteric nervous system (ENS). Cell also targeted the heart and small regularly sized balls of cells were a prominent feature of the meninges—both structures having a neural crest-derived component.[20,21] A very consistent but unexpected feature was the sizable clumps of cells located in the tail of the embryo. To understand why Kelly cells should target the tail, sections were stained with a neuronal marker, Tuj1. This revealed that the Kelly cells were loosely associated with the fine network of parasympathetic fibres found in this region. Kelly cells were integrated in the ciliary ganglion located behind the eye, thus confirming that they were clearly targeting the parasympathetic pathways (Figure 2). We also observed cells in the tongue and jaw regions and in the eye, particularly around the iris, but very few, if any, cells were observed in major organs such as the liver, lungs or kidney. Surprisingly, Kelly cells were never seen in the adrenal gland. To confirm that the Kelly cells had not fused with chick cells or been phagocytosed by, for example, chick macrophages, we confirmed that the GFP-expressing cells continued to stain with human specific anti-N-CAM (CD56) and NB84[22] and did not stain with chick specific anti-GAP-43 (Supplementary Figure S1).

For comparison, Kelly cells were also injected at E6, after neural crest migration was complete and cells targeted the liver and kidney (Figure 2). The meninges continued to be a favoured site but the cells were never seen in the SG or the adrenal gland. A few cells continued to migrate into the ENS possibly reflecting the sacral contributions to the developing ENS.[23] To test whether the change in localisation of the Kelly cells was due to evolving blood flow circuits between E3 and E6, fluorescent beads were injected at both time points and the major locations of the beads then analysed at E10. The distribution of the beads was essentially the same irrespective of when they were injected, suggesting the Kelly cells are indeed responding to different embryonic cues at E3 and E6 (Figure 2). Thus, we have established an attractive system by which to analyse the effect of different embryonic environments on the *MYCN*-amplified neuroblastoma cells.

Kelly cells respond differentially to neural and non-neural environments

Confocal analysis of Kelly cells within the different chick tissues revealed that Kelly cells within neural networks were integrated into the host tissue and showed morphological changes consistent with cells migrating or beginning to differentiate (Figures 3a–c). Cells often had an elongated morphology and some had extended short processes. They were either single or in groups of 3–4 loosely associated cells. Staining with anti-NF 70 kD revealed how the Kelly cells closely associated with host neurons, particularly in the ENS (Figures 3d and e). The Kelly cells themselves did not stain with NF 70 kD in either SG or ENS. Cells in non-neural environments typically formed aggregates consistent with them showing little migration and/or continuing to proliferate (Figures 3f and g). The results observed with the Kelly cells were confirmed with SK-N-BE(2)C cells, a second *MYCN*-amplified neuroblastoma cell line (Supplementary Figure S2).

To test the extent of Kelly cell proliferation in the different tissues, embryos were injected with EdU at E9 and sacrificed 24 h later. In SG, host cells continued to divide between E9 and E10 while the GFP Kelly cells frequently appear as an unlabelled region (Figure 4a) with just an occasional labelled nucleus. Overall 15% of

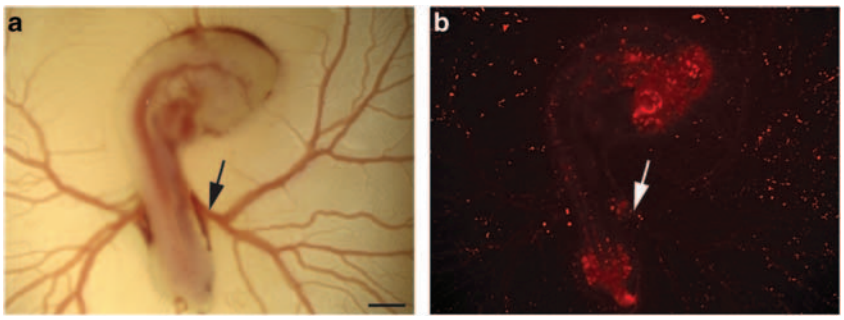

Figure 1. Kelly cells were injected into the extra embryonic blood vessels of an E3 (HH stage 18–20) chick embryo. Approximately 200 000 DiI labelled Kelly cells were injected in 2 μl volume, and the distribution of cells observed approximate 2 h following injection. (**a**) Bright field, (**b**) fluorescence image. Arrow indicates a typical site of injection. Cells could be observed circulating through the vitelline blood vessels for up to about 3 h following injection, although the majority of cells extravasated and colonised most regions of the embryo. Scale bar 1 mm.

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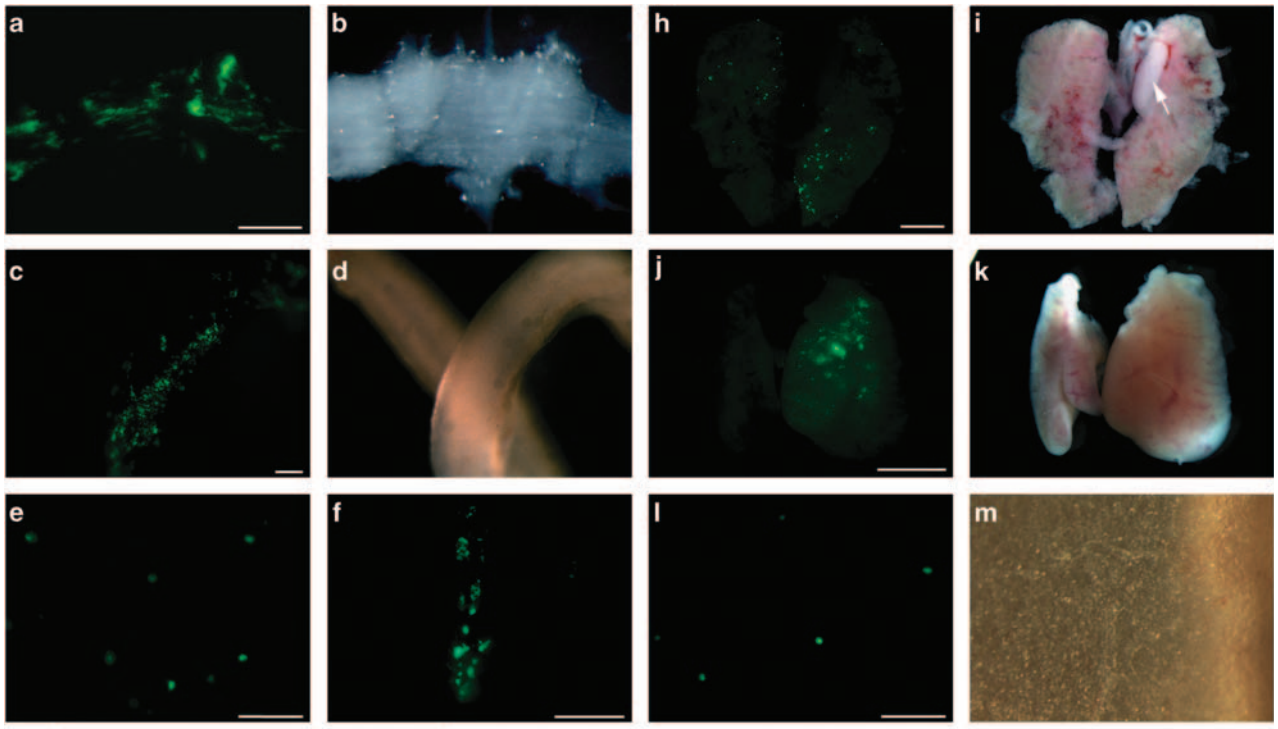

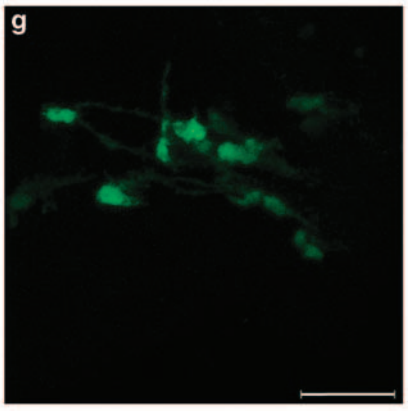

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Chick tissue	Kelly E3	Kelly E6	Fluo beads E3	Fluo beads E6
Symp. ganglia	+++	-	+	+
Gut	+++	+	+	++
Adrenals	-	-	+	+
DRG	+	-	-	-
Tail	+++	+	++	++
Meninges	+++	++	+++	++
Heart	++	+	+++	+++
Liver	-	+++	+++	+++
Kidneys	+	+++	+++	+++

Figure 2. Kelly cells target neural crest-derived tissues when injected at E3, but not at E6. (**a**–**g**) correspond to injections at E3 and dissection at E10. (**a**) Fluorescence and (**b**) bright field image of a sympathetic ganglion. Kelly cells are distributed throughout the ganglion. More than 95% of SG from successfully injected embryos contained >10 cells and many contained >100 cells (>100 embryos dissected). Scale bar 200 μm. (**c**) Fluorescence and (**d**) bright field image of the gut. Intermittent groups of cells could be observed along the gut in almost all embryos dissected. Scale bar 200 μm. (**e**) Fluorescence image of the balls of cells located in the meninges. The meninges contained variable numbers of these regularly sized balls, but typically about 100 were observed. Scale bar 200 μm. (**f**) Fluorescence image of clumps of cells observed in the tail region of embryos, associated with the fine parasympathetic network of fibres. These clumps, unlike the balls in the meninges, were variable in size. Scale bar 1 mm. (**g**) Confocal image of Kelly cells integrated into the ciliary ganglion. Scale bar 20 μm. (**h**–**m**) correspond to injections at E6 and dissection at E10. (**h**) Fluorescence and (**i**) bright field image of kidney. Note that Kelly cells injected at E6 still do not target the adrenal gland (indicated by arrow). Scale bar 1 mm. (**j**) Fluorescence and (**k**) bright field image of Kelly cells forming variable sized clumps in the liver. Scale bar 2 mm. (**l**) Fluorescence and (**m**) bright field image of meninges showing regular sized balls of Kelly cells. Scale bar 200 μm. (**n**) Relative distribution of cells or beads injected at E3 and E6 were estimated for various locations. The distribution of beads was similar at both ages, in contrast to the behaviour of the cells. Small numbers of beads did locate to the adrenal gland, confirming that the absence of Kelly cells was not due to a lack of vascularisation. Cells were seen occasionally along nerves and blood vessels and in other locations. They were never seen in the brain or spinal cord.

Kelly cells had divided in 24 h (Figure 2d). Similar results are seen for the Kelly cells in the gut (Figure 4d), while in the tail and meninges of embryos injected at E3 and analysed at E10 about 50% of Kelly cells are dividing (Figures 4b and d). The proliferation of Kelly cells in liver, meninges and kidney was analysed after 4 days *in vivo* (E6–E10), and as expected the clumps of Kelly cells were actively dividing with 35–50% labelled with EdU (Figures 4c and d). Thus proliferation and the formation of micro tumours was a consistent feature of an inappropriate embryonic environment despite cells in some cases following neural crest cells to the location of the parasympathetic network and becoming loosely associated with these parasympathetic fibres. Kelly cells embedded within the SG and ENS did appear to have their proliferation rate restricted, although cells did nevertheless continue to divide. This was in keeping with the host tissues in which cell division was also occurring.

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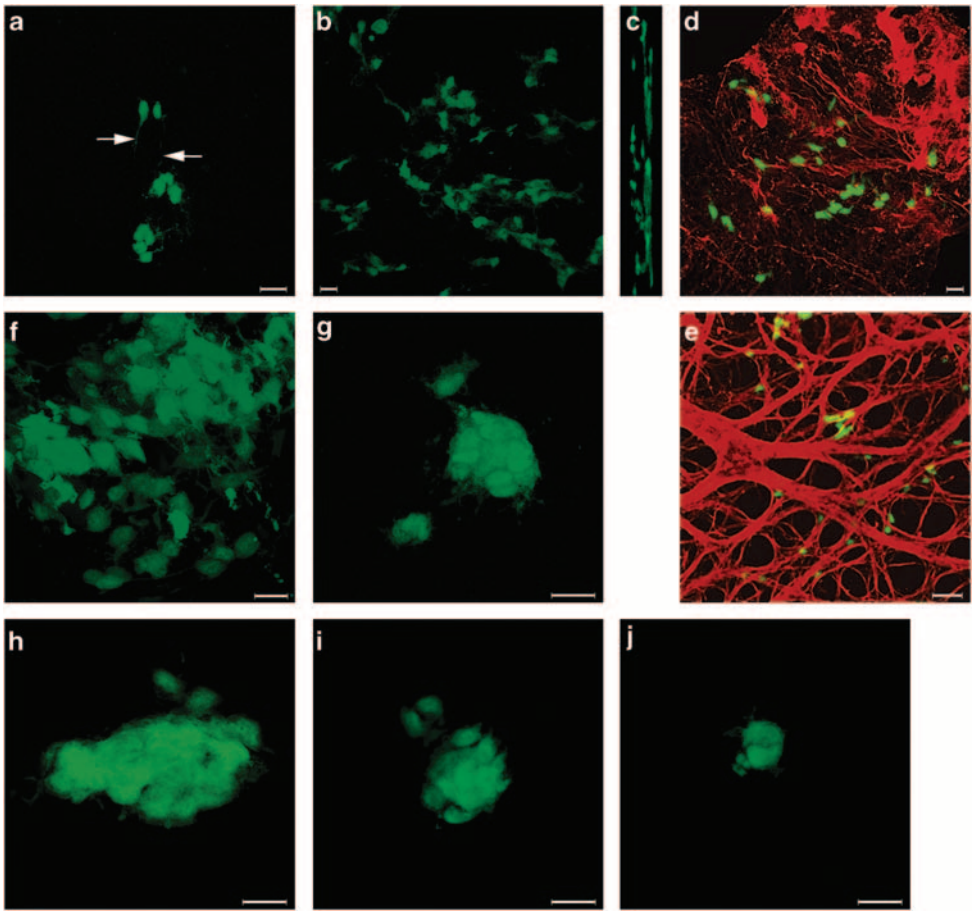

Figure 3. Kelly cells undergo morphological changes in response to different tissue environments at E10. (**a**–**g**) correspond to injections at E3. (**a**) Kelly cells integrate into SG and display a differentiating morphology (arrows point to processes). (**b**) Kelly cells in the gut. Short pieces of intestine were cut in half longitudinally, and mounted on a glass slide, luminal surface down. This image is taken from above, 'looking down' onto the Kelly cells within the ENS, whilst (**c**) is a 3D reconstruction of the same image rotated though 90°, revealing the Kelly cells integrated into the two layers of the ENS, the myenteric plexus and submucosal plexus. (**d**) SG were wholemount stained with anti-NF-70 that cross reacts with chick and human (red). Only the sympathetic neurons and their axons stain well, but the Kelly cells (green) are integrated into the ganglion and exhibit many short processes. (**e**) Gut was wholemount stained with anti-NF-70 and this highlights the ENS network (red). Again the Kelly cells (green) are integrated into the ENS, but fail to stain with the anti-NF-70. (**f**) Small piece of tissue from the tail of the embryo containing one or more clumps of Kelly cells was mounted and imaged. Cells were typically in close contact and clumps were of variable size, suggesting cells may have aggregated following arrival in the tail region. (**g**) Ball of cells from the meninges, note the cells are highly adherent to each other. (**h**–**j**) correspond to injections at E6. (**h**) Kelly cells form variable sized clumps in the liver. (**i**) They form smaller and more regular sized clumps in the kidney. (**j**) They form regular clumps in the meninges. Note the smaller size of the meningeal clumps compare with those formed 7 days post injection. All scale bars 20 μm.

Kelly cells in culture express high levels of MYCN and it was interesting to investigate whether the expression level of MYCN varied in Kelly cells in the differing chick embryo environments. Kelly cells in the tail, kidney, liver and meninges, all maintained a high level of MYCN expression, whereas, in contrast, MYCN expression was downregulated, below detectable levels, in all Kelly cells in the SG and ENS (Figures 4e and f and data not shown). This was a very consistent result seen in >50 embryos and in all Kelly cells in these locations and strongly suggested the Kelly cells in these areas had been reprogrammed to a less aggressive phenotype. It was therefore intriguing that 15% of cells in SG and ENS continued to divide.

Cell–cell contact with the SG is required to maintain downregulation of MYCN expression

Embryos injected at E3 were therefore allowed to survive to E14 to test whether cell division might be further reduced in Kelly cells in the SG following the complete loss of detectable MYCN expression. The overall location of the Kelly cells in the E14 chick embryos was similar to that at E10 with cells continuing to survive in all locations seen at E10. The morphology of a small number of Kelly cells in the SG was striking as the cells had extended long axons similar to the differentiating host neurons (Figure 5a). These cells also stained with the differentiation maker GAP-43 (Figure 5e). Some of the cells were observed in relatively small but more closely associated clumps. The dividing clumps in tail and meninges had expanded in size consistent with increasing time in the embryo. The balls of cells in the meninges have a diameter of 50–100 μm after 11 days compared with about 30 μm after 7 days and about 15–20 μm after 4 days *in vivo* (Figures 2 and 5c). Their regular size suggested each clump had arisen from a single cell. As shown in Figure 5, the proportion of Kelly cells within the SG and ENS continuing to divide had increased to 22% and 27%, respectively, whilst Kelly cells in the tail and meninges continued to proliferate as before. In both ENS

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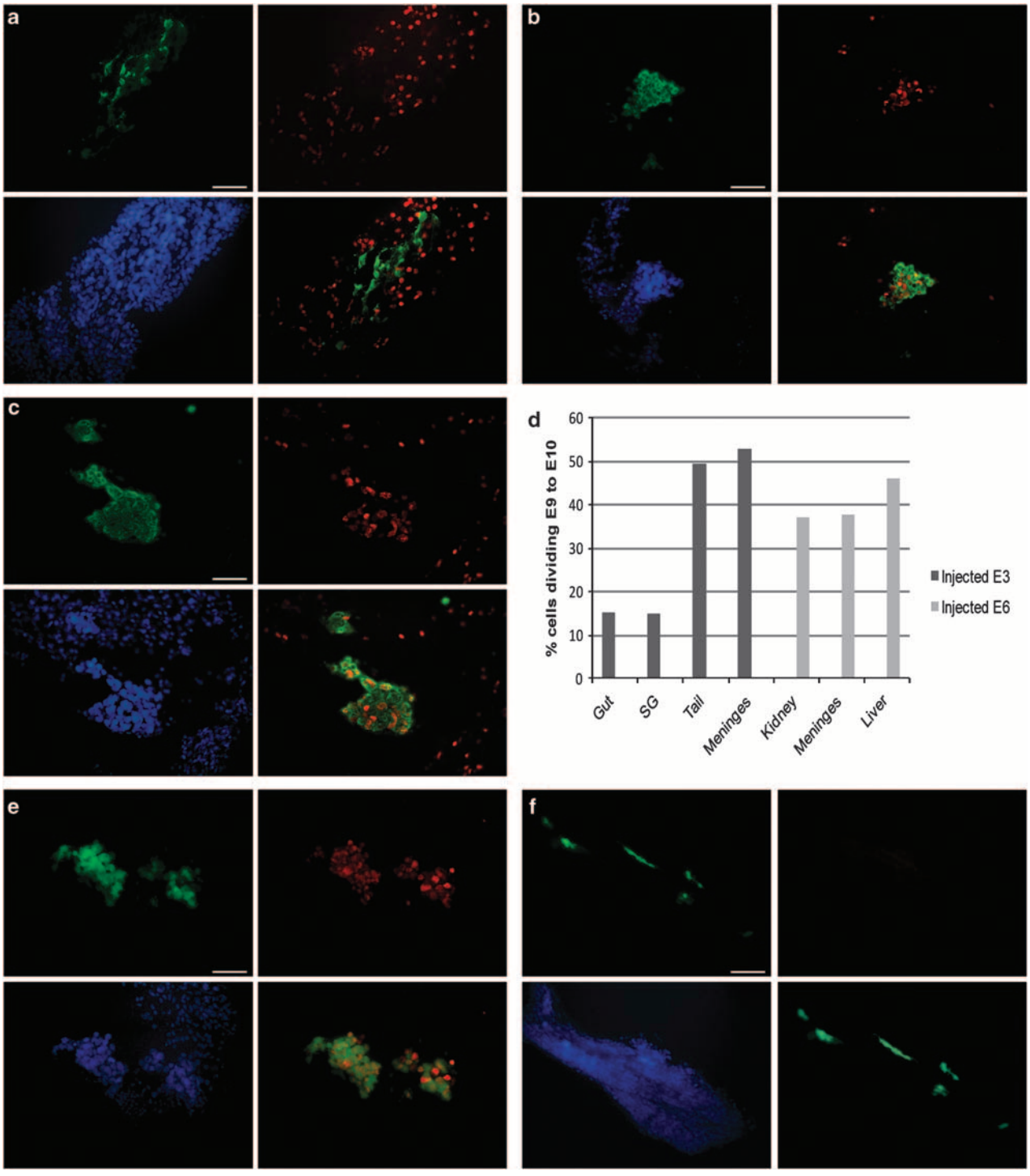

Figure 4. Cell division and MYCN expression in Kelly cells show differential responses according to their location in different tissues. (**a**–**c**) The results from embryos injected with Kelly cells at E3 (**a**, **b**) or E6 (**c**), injected with EdU at E9 and dissected at E10. Kellys were stained with anti-GFP (green), EdU incorporation detected for the entire tissue section (red), and all nuclei were stained with Hoechst 33342 dye (blue). Bottom right photograph in each panel is merged image of anti-GFP and EdU. (**a**) SG, the host chick tissue shows numerous dividing cells while the Kelly cells are largely unlabelled. (**b**) Tail, relatively few cells in the host tissue are dividing however large numbers of Kelly cells have incorporated EdU. (**c**) Liver, host cells are dividing however large numbers of Kelly cell have also incorporated EdU in the previous 24 h. (**d**) The proportion of Kelly cells dividing relative to the total number of Kelly cell nuclei were counted ($n = >180$). (**e**, **f**) The results from embryos injected with Kelly cells at E3 and dissected at E10. GFP Kelly cells are green, anti-MYCN stained nuclei are red, DAPI-labelled nuclei are blue and the bottom right quadrant shows a merged image of GFP and MYCN. (**e**) Tail, all Kelly cells clearly stained positive for MYCN. (**f**) SG, >100 integrated Kelly cells were viewed and none showed detectable staining for MYCN. All scale bars 50 μm.

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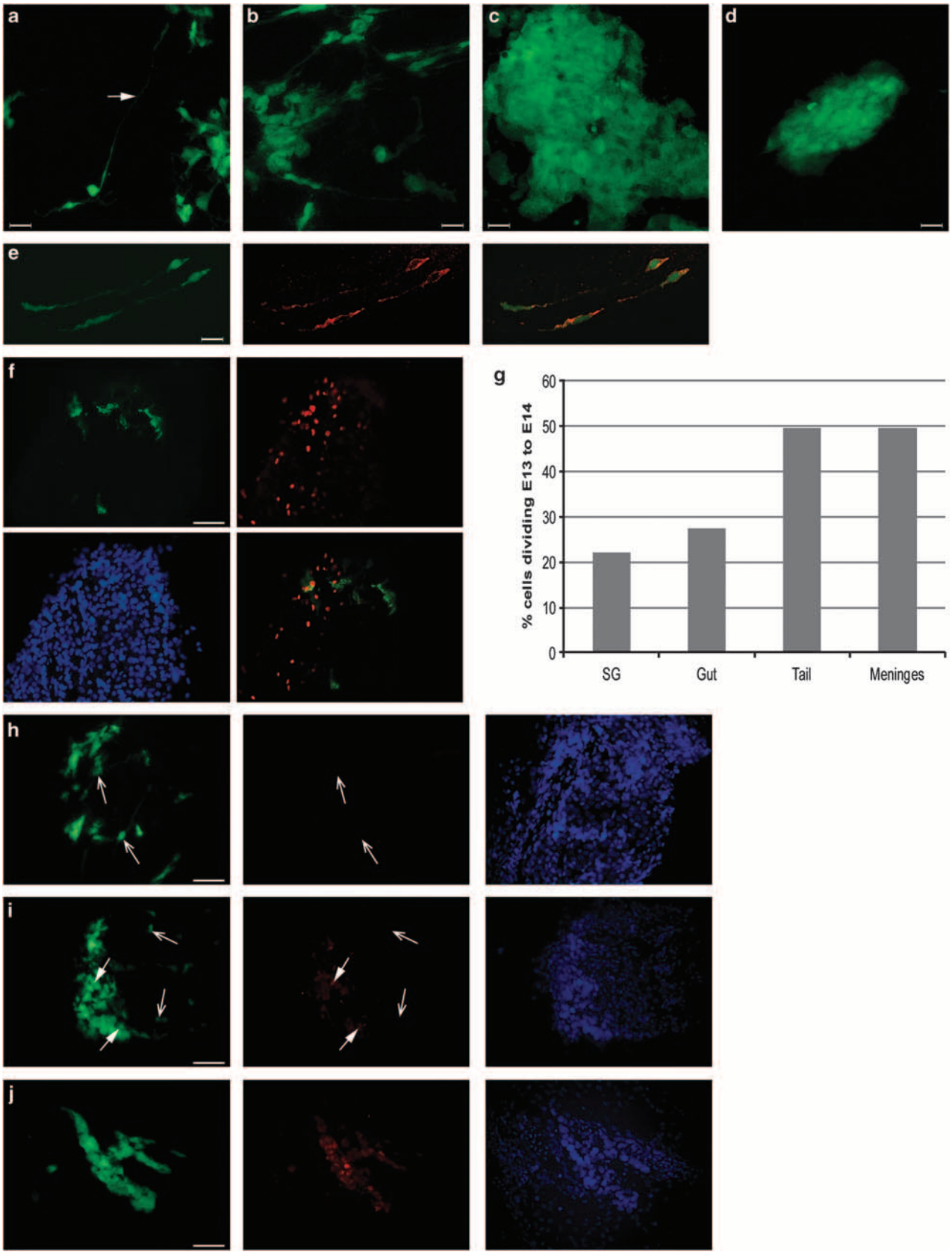
and SG, host chick cells were also dividing so this result was consistent with the Kelly cells continuing to respond to their environment.

The MYCN expression in Kelly cells in the SG and tail at E14 was also examined. Kelly cells in the tail continued to express MYCN as had been seen previously (Figure 5g). In the SG, the MYCN



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expression in single isolated cells remained undetectable in almost all the cells. However, cells in the small clumps, which were largely protected from the SG environment, did show re-expression of MYCN (Figures 5i and j).

To investigate further the requirement for contact with SG cells, the SG and the tail region was dissected and dissociated cells placed in culture (Figure 6a). MYCN expression persisted in the Kelly cells isolated from the tail and most cells were dividing. In Kelly cells dissociated from the SG, most cells had re-expressed MYCN within 3 days even when cells were clearly in contact with sympathetic neurons or their axons (Figure 6b). Their morphology was consistent with undifferentiated Kelly cells in culture. In contrast, when SG explants were placed in culture, MYCN expression remained undetectable and occasionally the Kelly cells were seen to extend long neurites in keeping with the host sympathetic neurons. Kelly cells in these explants were typically integrated into the ganglia and were surrounded by sympathetic cells. Many Kelly cells nevertheless underwent cell division, as did a surprisingly large number of host SG cells compared with the number dividing in SG *in vivo*. Thus, the Kelly cells appeared to remain under the influence of the SG even when returned to culture.

DISCUSSION

The chick embryo has a long history of being used to analyse tumour cell biology. The application of tumour cells to the chorioallantoic membrane is a well established model for investigating angiogenesis and metastasis.[24–26] More recently implanting of tumour cells early in development has been utilised to test whether the embryonic environment can influence the phenotype of malignant melanoma cells.[7,8] Relatively small numbers of cells are introduced (<2000 cells) and a proportion of these migrate and their phenotype is analysed over short-time periods. Results from these experiments were encouraging in that the melanoma cells detected neural crest migration cues and targeted SG irrespective of whether neural crest cells were present. Most recently these seminal findings have also been replicated in mouse embryos.[27]

Here we have developed a system whereby we can introduce larger numbers of cells ($\sim 200\,000$) and we find that cells, injected at E3, respond to neural crest guidance cues. This was initially surprising as the Kelly cells were not placed within the neural crest or along the normal migratory pathways. Rather some of the Kelly cells following extravasation, must have been attracted to the neural crest pathways. Melanoma cells survive, migrate and/or proliferate quite well when introduced to an inappropriate embryonic environment principally the chick eye cup.[9,28] Kelly cells did not survive well in the eye cup environment; however, injection of cells at E6 results in the cells targeting major organs principally liver and kidney where they proliferate and form microtumours. The targeting we have observed here is distinctive to *MYCN*-amplified neuroblastoma cells with cells from other tumours and embryonic stem cells targeting their own distinctive locations. One point of particular interest is the lack of targeting of Kelly or BE2C cells to the adrenal gland in the embryos. This is surprising as neuroblastoma arises from both SG and adrenal glands, and both structures are thought to differentiate from cells that were initially indistinguishable and have undergone terminal differentiation according to their final location.[29] More recently, it has been proposed that presumptive chromaffin cells that never express NF-M or SCG10 are already forming a distinct cell population by E5.[30,31] Nevertheless, Kelly cells injected at E3 clearly have the opportunity to migrate into the adrenal anlagen but instead exclusively select the adjacent nascent SG. Neuroblastoma tumours do not express phenylethanolamine N-methyltransferase (PNMT), a marker of chromaffin cells, leading to the proposal that tumours formed from the adrenal gland are derived from misplaced sympathetic neuroblasts rather than chromaffin progenitor cells.[32,33] If true, this may explain why Kelly cells and BE2C cells never choose to associate with the developing adrenal gland. In contrast, neuroblastoma cells do however target mature adrenal glands when injected intravenously into nude mice.[34]

Kelly cells show reduced cell division in neural tissue compared with non-neural tissues at both E10 and E14. This could be due to an overall decrease in cell cycle or the development of two populations of cells, some dividing and some not. Cells may cease cell division either temporarily because they are migrating or permanently because of differentiation. At E10, cells within the SG or ENS may be migratory as they do not at that stage express differentiation markers; however, by E14 as judged by morphology and GAP-43 expression, some cells are beginning to differentiate. Nevertheless, loss of MYCN at least in the short term does not result in the loss of proliferative potential. The consequences of downregulating MYCN expression in cultured cells have been investigated by a number of groups because controlling MYCN expression is an attractive potential treatment for *MYCN*-amplified tumours. Suppression of MYCN expression following transfection with miRNAs into Kelly cells inhibited proliferation and clonogenic growth,[35] while both Kelly and BE2C cells showed morphological changes consistent with differentiation and an overall reduction in cell proliferation.[36] These results are compatible with our *in vivo* findings. In addition, Henriksen *et al.*[36] found that expression of neurofilament and GAP-43 was upregulated within 3 days of knocking down MYCN expression. This was assayed by western blotting, so it is unclear whether their expression levels were sufficient for immunofluorescence detection.

One recently reported consequence of the loss of MYCN expression is the unregulation of TrkA and p75.[16] TrkA and p75 are markers of good prognosis for neuroblastoma so the possibility that these might be expressed as a consequence of MYCN downregulation is exciting and may lead to further changes in cell fate.

The concentration of MYCN protein in cells can be regulated by transcriptional activity, stability of the mRNA and protein degradation. Protein stability is controlled by a number of signalling pathways including mTOR,[37] whereas mRNA stability may be dependent on a variety of miRNAs and proteins including HuD and members of the p53 family.[35,38–40] However,

Figure 5. Kelly cells continue to divide in SG and the ENS at E14 and MYCN expression remains undetectable, except in cells protected from the SG environment. Embryos were injected with Kelly cells at E3, injected with EdU at E13 and dissected at E14. (**a**) Morphology of Kelly cells in SG. Note one cell with an exceptionally long axon (arrow). (**b**) Morphology of Kelly cells in ENS. (**c**) Morphology of Kelly cells in tail. (**d**) Morphology of Kelly cells in meninges, note increase in size of clump after 11 days in embryo compared with 7 days. (**e**) Kelly cells in an E14 SG showing GFP fluorescence (green), GAP-43 staining (red), and the merged image. (**f**) A small proportion of Kelly cells (green, anti-GFP) located in the SG (all cell nuclei stained with Hoechst 33342, blue) continue to incorporate EdU (red). Note that there are still dividing cells in the host SG. Bottom right is merged image of anti-GFP-stained Kelly cells and EdU-containing nuclei. (**g**) Summary of the numbers of dividing cells in the different tissue locations. Cells in a neural location show reduced cell division compared with a non-neural location. (**h**–**j**) Frozen sections of SG (**h**, **i**) and tail (**j**). GFP Kelly cells are green, MYCN staining is red, and all cell nuclei are stained with DAPI, blue. (**h**) No MYCN staining was typically detected where cells were mostly isolated within the SG ($n>50$). (**i**) MYCN is typically detectable in Kelly cells protected from the SG environment, arrows = no MYCN stain, block arrowheads indicate cells with detectable MYCN expression. (**j**) All Kelly cells within the tail had detectable MYCN expression.

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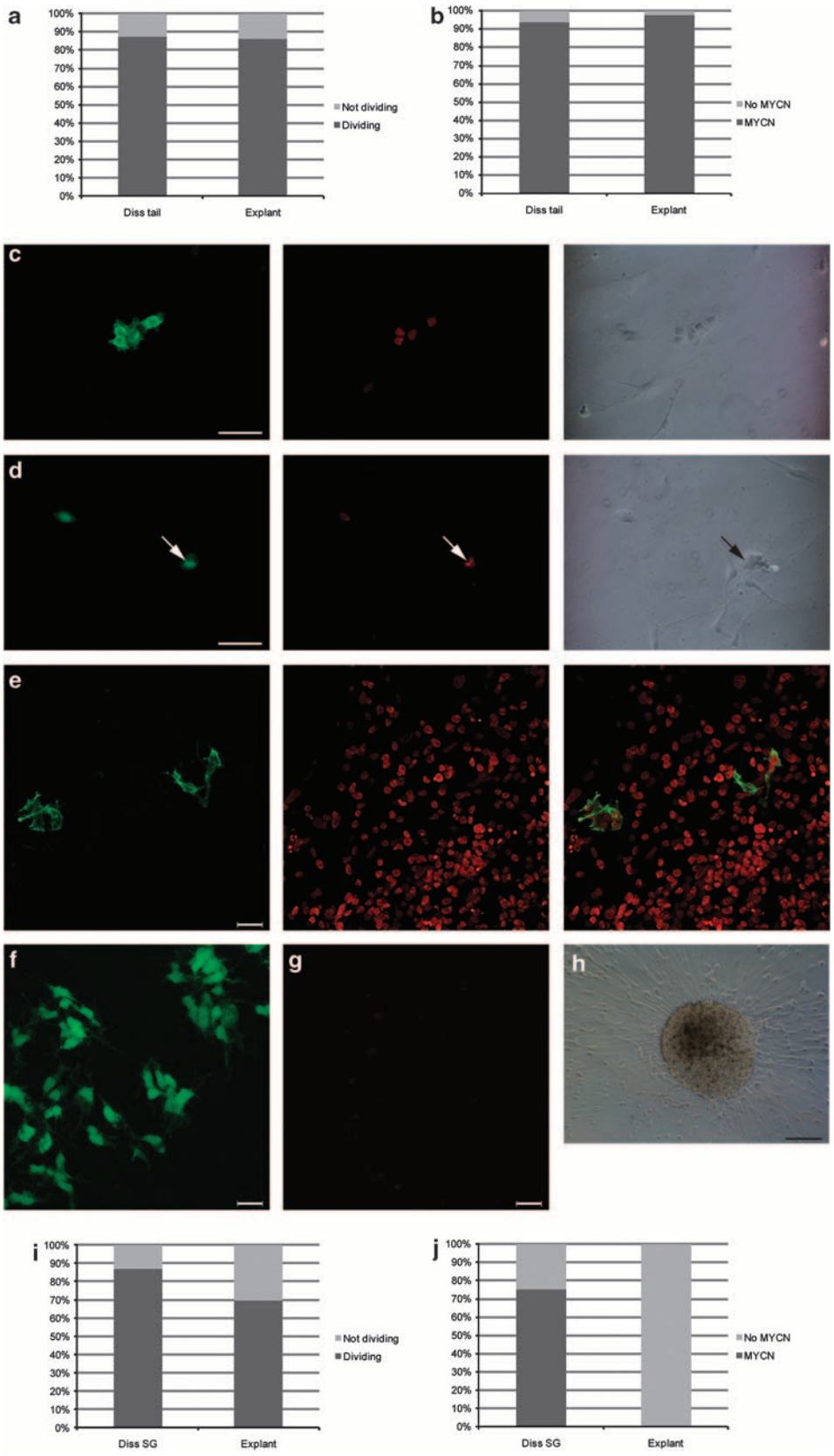
developmentally, MYCN expression will be controlled by suppression of transcription and this is likely to be the principal mechanism by which MYCN is downregulated in the Kelly cells, although a contribution by factors regulating mRNA and protein stability cannot be ruled out. The key factors that initiate suppression of MYCN expression remain to be elucidated. The



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regulation of MYCN expression is dependent on auto regulation[41] and E2F1, SP1, and SP3 have all been implicated in activating MYCN expression.[19,42] Downregulation of MYCN may depend on retinoic acid, vasoactive intestinal peptide (VIP) and nitric oxide (NO),[43–45] and bone morphogenetic proteins (BMPs) are known to downregulate MYCN expression via TEIG-1(KLF10) in cerebellar granule cell precursors.[46] The regulation of MYCN expression is also complicated by the presence of an antisense gene *N-CYM* located on the opposite strand.[47] This gene is co-expressed with MYCN and could in principle regulate the stability of processing of MYCN mRNA. The origin of the embryonic signals that regulate MYCN is also unclear. MYCN expression is turned off as neural crest cells differentiate into glial cells, and slightly later as they differentiate into neurons.[17] The signals that prompt this change may be present along the neural crest migration pathway and/or within the SG. The migration pathway alone seems to be insufficient as MYCN expression remains turned on in Kelly cells in the tail and meninges, while it remains to be determined whether the SG environment alone is sufficient to downregulate MYCN expression.

As Kelly cells respond to the chick embryo environment, we propose that the regulatory DNA sequences that respond to developmental cues are included in the amplified region. Kelly cells have an amplicon size of 953 kB[18] and because the Kelly cells require continuous signals from the SG environment to maintain MYCN downregulation, the DNA within the amplicon may be lacking features that enable a switch to a non-transcriptional state.

In conclusion, we have developed a model in which *MYCN*-amplified neuroblastoma cells are targeted to the SG and are reprogrammed to a more benign phenotype by the embryonic environment. Crucially all Kelly cells respond by downregulating MYCN, suggesting that *MYCN* amplicons may have sufficient regulatory DNA to respond to appropriate negative transcriptional controls.

MATERIALS AND METHODS

Cell culture

Kelly cells were transduced with an enhanced green fluorescent protein (EGFP)-expressing lentivirus. EGFP was expressed constitutively under the control of the spleen focus-forming virus promoter[48] and the viral particles were pseudo-coated with a vesicular-stomatitis-virus glycoprotein envelope.[49] Cells were cultured in RPMI 1640 medium containing 10% fetal calf serum (Biosera, East Sussex, UK), 100 U/ml penicillin and 100 μg/ml streptomycin, at 37 °C and 5% CO_2, in a humidified incubator. GFP-labelled SK-N-BE(2)C (kindly donated by Anne Herrmann) were cultured in the same way, but in Dulbecco's modified Eagle medium medium, supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin.

Neuroblastoma cell injections

Fertilised white leghorn chicken eggs (obtained from Lees Lane Poultry, Wirral, UK) were incubated at 37 °C and 35% humidity. Eggs were windowed at E2 or E3 (HH stage 12–18), following the removal of 3 ml of albumin to detach the embryo and surrounding vessels from the shell, and windows resealed using Scotch Magic adhesive tape. Before injection, GFP-labelled Kelly cells were harvested from culture flasks using 0.05% trypsin, counted and following centrifugation at 1000 *g* to remove the trypsin, resuspended as single cells at a density of 1×10^5 cells/μl in RPMI 1640, containing 100 μg/ml DNase I and 0.05% fastgreen. GFP-SK-N-BE2C cells were harvested as above with the exception that 1% collaginase was frequently added to ensure the production of a single cell suspension. In the earliest experiments, non-GFP-labelled SK-N-BE(2)C cells were harvested as above, incubated for 30 min with 40 μM Vybrant DiI (Invitrogen, Paisley, UK), and then resuspended at 1×10^5 cells/μl, as single cells in Dulbecco's modified Eagle medium containing 0.05% fastgreen. During injections cells were kept on ice and occasionally re-triturated with the addition of fresh DNAse 1. Green fluorescent beads were suspended in phosphate-buffered saline containing 0.05% fastgreen, again, at a density of 1×10^5 beads/μl. Borosilicate glass capillary tubing (thin wall with filament, Harvard Apparatus Ltd., Kent, UK) was pulled under heat to a thin taper, and the resulting needles snapped to an appropriate diameter—thin enough for microinjection, but wide enough to allow entry of the cell suspension. Suction was applied via mouth pipette to fill the needle with 2 μl of the appropriate cell suspension, and this was then injected, under a stereo microscope, into the extra-embryonic vitelline vein of each embryo at E3 (Figure 1) or E6. Embryos were incubated until E10 or, for some E3 injections, until E14. To assess cell proliferation typically 1 μl of 20 mM EdU (Click-iT EdU imaging kits, Invitrogen C10339) was injected into an extra-embryonic blood vessel at E9 for 24 h, although 0.5 and 2 μl was injected with no appreciable difference in the percentage of cells labelled. 4 μl of 20 mM EdU was injected at E13. Embryos were then dissected 24 h later (at E10 or E14), under a Leica M165 FC fluorescence microscope, and locations of GFP-labelled cells noted and photographed. Tissues-containing GFP-Kelly cells were then fixed using 4% paraformaldehyde.

E10 SG and tail culture

Following dissection of the E10 embryos, SG and tail tissue, both containing GFP-Kelly cells, were incubated for 30 min in 0.25% trypsin, dissociated by mechanical trituration, and plated onto laminin-coated coverslips in L15 medium, containing 0.5% methyl cellulose, 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.3% glucose and 5 ng/ml nerve growth factor. Other ganglia and tail tissues were torn into small pieces and plated as explants onto laminin-coated coverslips, in L15 medium as described above. These were incubated at 37 °C in a CO_2-free incubator for 48 h, after which time 5 μM EdU was added to the culture medium in each well, and cells were then incubated for a further 24 h before fixation in 4% paraformaldehyde.

Immunofluorescence staining of frozen sections/dissociated cells

Fixed tissues were sequentially placed in 6, 12, then 18% sucrose for 1 h each. The tissues were then mounted on a cork disc in Cryo-M-Bed embedding compound (Bright, VWR International Ltd., Leicestershire, UK) and frozen in isopentane held over liquid nitrogen. Ten micrometre thick frozen sections were then cut, and those containing GFP-Kelly cells were stained using antibodies to MYCN (1:10, ab16898, Abcam, Cambridge, UK),[50] CD56 (1:2.5, 852.703.020, Diaclone, Oxfordshire, UK) and NB84 (1:200, NCL-NB84, Novocastra, Milton Keynes, UK). EdU incorporation into GFP-Kelly cell nuclei was detected by following the Invitrogen protocol, also incorporating a step of GFP detection using rabbit-anti-GFP antibody (1:500, ab290, Abcam). The percentage of dividing cells was determined by counting EdU-labelled cells as a percentage of GFP-labelled cells. A minimum of 180 cells from two experiments including tissue from at least three

Figure 6. MYCN remains undetectable in Kelly cells in explanted SG but is re-expressed when Kelly cells and sympathetic cells are dissociated and placed in culture. As a control, cells from the tail region of embryos were dissociated and placed in culture, and clumps of Kelly cells along with surrounding tissue were explanted. The cultures were labelled with EdU after 2 days and stained for EdU incorpoation and MYCN expression after 3 days. (**a**) Shows that almost all Kelly cells are dividing in both explants and dissociated tail cultures, while (**b**) shows that almost all Kelly cells stain for MYCN. (**c** and **d**) SG dissociation culture. Scale bars 50 μm. (**c**) Kelly cells (green) are dividing (EdU incorporated, red), and (**d**) Kelly cells (green) have re-expressed MYCN (red), even when cells are still in contact with sympathetic neurons (see arrowed cell). (**e** and **f**–**h**), SG explant culture. (**e**) Shows the surprising increase in cell division within the host ganglia—EdU-labelled nuclei are red, anti-GFP stained Kelly cells are green, and the third (merged) image displays presence of some dividing Kelly cells. Scale bar 20 μm. (**f**) GFP Kellys within an explanted piece of SG, (**g**) remarkable lack of MYCN expression (red) in those same Kelly cells incorporated in the SG explant. Scale bar 20 μm. (**h**) Phase image of SG explant, scale bar 50 μm. (**i**) Bar chart of dividing vs non-dividing Kelly cells in SG-dissociated cultures and explants. Almost 90% have divided in the previous 24 h in culture whilst almost 70% have divided in the explants ($n = >150$ cells). (**j**) Bar chart of MYCN positive Kelly cells in dissociated cultures and explants. More than 70% of Kelly cells have re-expressed MYCN in dissociated cultures, no Kelly cells within the explants had detectable MYCN staining ($n = >150$ cells).

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embryos per experiment was analysed. Confocal images of GFP Kelly cells in tissues were obtained by dissecting out small pieces of tissue containing fluorescent cells, fixing them in 4% paraformaldehyde and mounting between a slide and coverslip in Dako fluorescent mounting medium.

Wholemount/explant immunofluorescence staining

Fixed tissue pieces were incubated overnight at 4°C in primary antibodies to MYCN (1:10, ab16898, Abcam) rabbit polyclonal NF70kD (1:500), chick-specific GAP43 (1:500),[51] and human-specific GAP43 (1:1000, ab75810, Abcam). Tissues were thoroughly washed in 0.5% Triton-containing phosphate-buffered saline over the course of 6 h, and then incubated overnight at 4 °C with the appropriate secondary antibodies (1:500, Invitrogen, Alexa 568/594).

Confocal imaging

Tissue pieces mounted onto microscope slides were viewed and photographed on a Zeiss LSM 710 confocal microscope.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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