https://theses.gla.ac.uk/

Theses Digitisation:

https://www.gla.ac.uk/myglasgow/research/enlighten/theses/digitisation/

This is a digitised version of the original print thesis.

Copyright and moral rights for this work are retained by the author

A copy can be downloaded for personal non-commercial research or study, without prior permission or charge

This work cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given

Enlighten: Theses
https://theses.gla.ac.uk/
research-enlighten@glasgow.ac.uk

THE ROLE OF CELL-TO-CELL INTERACTIONS IN THE SUPPRESSION OF THE TRANSFORMED PHENOTYPE

Iain W. Foulkes

This thesis is submitted in part fulfilment of the degree of
Doctor of Philosophy
in the University of Glasgow

November 1996

Beatson Institute for Cancer Research,
CRC Beatson Laboratories,
Bearsden, Glasgow.

Faculty of Medicine,
University of Glasgow,
Glasgow.

© I. Foulkes 1996

ProQuest Number: 10992198

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.

ProQuest 10992198

Published by ProQuest LLC (2018). Copyright of the Dissertation is held by the Author.

All rights reserved.
This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Thesis
10766
Copy 1

GLASGOW
UNIVERSITY
LIBRARY

ABSTRACT

The growth and aberrant morphology of certain transformed cells can be suppressed when cultured in the presence of excess resting normal cells. This well established phenomenon has been correlated with the presence of heterologous gap junctional intercellular communication, (GJIC) between the two cell types and the inhibition observed may provide an important defence against cancer. This thesis describes a study into this potentially important form of growth inhibition.

For this project a new assay was developed which enabled this form of inhibition to be studied in detail. The transformed cells were tranfected with a β-gal lineage marker to allow identification in mixed culture with normal cells. The proliferative status of both the normal and transformed cells was determined using a growth assay based on thymidine incorporation and analysis by autoradiography. A series of normal and transformed cell lines was selected with a range of homologous and heterologous communication phenotypes and these were used to assess the role of junctional communication in the inhibition phenomenon.

Standard focus assays, in which transformed cells are cultured with excess, growth arrested, normal cells, were performed to determine the extent of transformed cell growth inhibition by normal cells. The majority of foci which formed were markedly smaller than respective control colonies and this size suppression showed a positive correlation with heterologous communication. However, further analysis revealed that in many instances focus compression, mediated by the physical presence of the normal cells, significantly contributes to the explanation of focus size inhibition. Furthermore, the level of proliferation in some small foci was very high and would suggest that some transformed cells are lost from the culture dish due to high cell density coupled with a defect in cell-substrate adhesion. The results show that the use of focus size as an index of growth can generate inaccurate measurements of growth suppression.

Transformed cells can be inhibited by resting normal cells by a mechanism consistent with transfer of inhibition via gap junctions. However, the data appears to show that the presence of heterologous communication between normal and transformed cells is not necessarily sufficient for inhibition to occur and other factors may be involved. It was also shown that inhibition occurred in the absence of detectable communication.

The growth of normal cells can be significantly inhibited when they are co-cultured with excess transformed cells and this does not appear to be due to nutrient deprivation. The data suggests that a high proportion of the normal cells have competed with each other and with the transformed cells, which they recognise as an 'edge', for the amount of space they require to grow and divide, and as a consequence, become growth inhibited. It would appear that two different inhibition phenomena exist, one which may involve the transfer of inhibition from resting cells to growing cells and one which is, at least in part, due to contact-inhibition or the ability of cells to respond to the physical presence of other surrounding cells.

A higher proportion of normal cells (relative to controls) surrounding the majority of foci were found to be dividing. The level of growth stimulation decreased as distance from the focus periphery increased and there appeared to be a wave-like pattern to the distribution of stimulated cells. The stimulation may be caused by the disruption of the contact-inhibition mechanism, which

could be responsible for some of the inhibition observed in normal cell colonies surrounded by excess, transformed cells. This disruption may be caused by the foci expanding and the transformed cells competing for space on the culture dish. Normal cells in direct contact with the transformed cells, were less likely to be stimulated than cells 2-3 cells away, a pattern that may result from contact-inhibition by the transformed cells.

The inhibition of transformed cells by normal cells is more complex than is often suggested and more than one mechanism may be involved. A new inhibition phenomenon has been identified which appears to be mediated by contact-inhibition and may be related to the ability of cells to compete for space on the culture dish. Data from this study has highlighted the need to assess the growth of cells directly rather than using indirect growth indices such as focus size. The assay developed for this study provides detailed and accurate information on cell growth and could be used to look for an inhibition phenomenon *in vivo*.

for my family and Lindsey

TABLE OF CONTENTS

CHAPTER TWO

MATERIALS & METHODS.

CHAPTER THREE

RESULTS

Acknowledgments

I would particularly like to thank Dr. John Pitts for all his help and supervision. I would also like to thank all members of R5, past and present, for there help in the lab and Professor F. Tato for the cell lines. I would like to thank my parents, family and friends for their help and encouragement over the course of the project. Special thanks to Lindsey for putting up with me and putting me up. Finally I would like acknowledge the financial support of the Medical Research Council and Cancer Research Campaign.

Declaration: The work described in this thesis was performed personally unless otherwise acknowledged.

Abbreviations

β-gal	β-galactosidase
BSA	Bovine serum albumin
Ca^{++}	Calcium
cAMP	3', 5'-cyclic adenosine mono-phosphate
CDK	Cyclin dependent kinase
CM	Conditioned medium
Cx	Connexin
DEPC	Diethyl pyrocarbonate
DMEM10%	Dulbecco's Modified Eagle Medium supplemented with 10% FCS
DMSO	Dimethylsulfoxide
EC-cells	Embryonal carcinoma cells
ECL	Enhanced chemilluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetra-acetic acid
EGF	Epidermal growth factor
FAK	Focal adhesion kinase
FCS	Foetal calf serum
FITC	Fluorescein-isothiocyanate
G418	Geneticin
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GJIC	Gap junctional intercellular communication
HEPES	N-[2-hydroxyethyl]-piperazine-N'-[2-ethanesulfonic acid]
hp	Horseradish peroxidase
Ig	Immunoglobulin
IP_3	Inositol triphosphate
kDa	Kilodalton
LI	Labelling index
MAb	Monoclonal antibody
MAP kinase	Mitogen activated protein kinase
MOPS	4-morpholinpropanesulfonic acid
MW	Molecular weight
NCAM	Neural cell adhesion molecule
PAG	Polyacrylamide gel
PBS	Phosphate buffered saline
PDGF	Platlet derived growth factor
SatD	Saturation density
Sd	Standard deviations
TPA	12-0-tetradecanoylphornol-13-acetate
TdT	Terminal deoxynucleotidyl transferase

[^{3}H]-TdR	Tritiated thymidine
TUNEL	Terminal deoxynucleotidyl transferase nick end labelling
v/v	Volume to volume
w/v	Weight to volume
X-gal	5-Bromo4-Chloro3-Indoyl b-D-Galacotpyranoside
TGF	Transforming growth factor
TK	Thymidine kinase

List of Figures *pages following*

List of Tables *pages following*

CHAPTER ONE

INTRODUCTION

1.1. General Introduction.

The evolution of eucaryotic cells is defined by the development of a nucleus and the diversification of cellular organelles. Cells increased in complexity as the size of the nuclear genome increased which, together with genetic recombination, permitted cells to express their hereditary information in many different ways. The onset of multicellularity allowed for cellular diversification and specialisation which ultimately led to the development of distinct tissues and organs. A principle advantage bestowed on a multicelled organism is its ability to exploit resources, in a way that no single cell can. This is ultimately achieved by the co-operation and co-ordination between the cells which form the organism.

Co-ordination between the cells of a multicellular organism is fundamental to its development and success within its environment. This is achieved by various communication mechanisms which are employed to varying degrees at different stages of development. Essentially three modes of communication amongst animal cells have been discovered to date:

1). Signalling by secreted factors; these signals effect cells close to or distant from the signal source and include endocrine, paracrine and synaptic signalling (Alberts et al 1989 for review).

2). Juxtacrine or adhesion-mediated signalling, in which certain cells displaying membrane-bound growth factors or adhesion receptors bind to and influence other cells displaying appropriate ligands on their cell surface (Rosenburg & Massague 1993)

3). Gap junctional intercellular communication (GJIC) in which cells are connected (coupled) by transmembrane channels that allow the movement of small molecules less than 1kDa in size. Thus cells within a tissue or organ are able to give a co-ordinated response to a signal which only a few cells may have initially received.

Cohesion between the communicating cells is critical and is maintained by a series of cell-cell junctions such as tight junctions (which serve as a physical barrier responsible for separating interstitial cells from other cells within a tissue), adherens junctions, desmosomes in vertebrates and septate junctions in invertebrates.

Although the communication mechanisms outlined above vary in terms of distance, type of signalling molecule and mechanics of signal transmission they are all closely linked in terms of achieving cellular co-ordination and in relationship to each other. Effector molecules such as hormones and growth factors induce cascades of cytoplasmic events following initial signal transduction (Alberts et al 1989). The second messengers such as IP_3 and Ca^{++} which are activated during growth factor stimulation can rapidly spread through gap junctions resulting in a co-ordinated response to a given stimulus (Saez et al 1989, Stauffer et al 1993). Juxtacrine and GJIC mediated signalling are very localised and require cell to cell adhesion, they therefore provide a means of

integrating responses to more distant signalling systems; although at present poorly understood, such interactions may prove to be important in tissue morphogenesis.

Of particular importance in development are the mechanisms by which signals generated by one group of cells controls or determines the fate of another population of cells. In early embryonic development this process is termed embryonic induction and is one important mechanism for generating differences between cells. The existence of even two cells offers the potential for communication. If those two cells differ, for example, in there cytoplasmic constituents then the potential exists for inductive interactions to take place (Greenwald & Rubin 1995). In embryonic development interactions between mesenchym and epithelial cells eventually leads to the subsequent development of different cells and tissues (Hay 1981). As the organism develops, interactions on a long range basis become important, these are mediated by the hormonal, neural and vascular systems. Throughout the developmental process the cell surface and associated extra cellular matrix (ECM) assume pivotal roles in translating external signals into altered gene activity (Gimond & Au mailley 1992). Adhesive interactions between cells and the ECM are crucial in many stages of embryonic morphogenesis (for review see Juliano 1993a) and ultimately lead to the translation of basic genetic information into complex 3-dimensional tissues and organs.

Mediated by the types of cell-cell interactions described above, the extracellular environment can exert a profound effect on cell phenotype. Embryomal carcinoma cells provide a striking, *in vivo* example where environment influences gene expression. These malignant cells can undergo phenotypic reversion when placed in a normal tissue environment of a blastocyst. This phenomenon may provide a suitable analogy to the phenomenon observed by Stoker and others in which the aberrant growth of transformed cells is abrogated when cultured with excess normal cells (Stoker 1967; section 1.6). The potential role of gap junctional communication as a mediator of this growth control is considered below. A detailed review of the role of GJIC in normal growth and developmental systems is provided followed by an examination of the evidence concerning its part in the process of carcinogenesis. Finally, gap junctional communication is considered as a potential mechanism for regulating cellular phenotypes within the context of the Stoker observations (Stoker 1967; see above). The aims of the project are provided in section 1.7.

1.2. Cell-cell interactions and their role in the determination and maintenance of cell phenotypes.

1.2.1. The cell surface: a primary interface of all cell-cell interactions.

The cell surface is the first point of contact for many of the extracellular signals responsible for influencing, to a large extent, the phenotype of a particular cell. Such signals may be hormonal, ionic, the proximity of other cells, cell shape and ECM products. Included in the cell surface are structures which link cells together for example adhesion molecules and gap junctions, their role in influencing cell phenotype is discussed below.

1.2.2. Secreted factors.

There is a wide variety of secreted factors involved in the regulation and development of cells. They include hormones and other diffusible factors (morphogens). Hormones may bind to a receptor on the cell surface and induce a cascade of secondary signalling events. Others pass through the membrane and influence gene expression more directly e.g. steroid hormones. Many diffusible factors are involved in inductive signalling (a signal emanating from one cell which controls gene expression in a responding cell), for example, the TGF, HGF/SF, Hedgehog, FGF and Wnt families of secreted proteins. These are thought to be of particular importance during embryonic induction (Jessel & Melton 1992) and mesodermal differentiation (Slack et al 1987, Asashima et al 1990, Christian et al 1991, Ingam 1994, Joannou et al 1995, Bradley & Brown 1995). Longer-range inductive signals involving the above molecules are thought to be responsible for the control of patterning in several embryos. However, the way in which they achieve such a distant organising activity is unknown. There are currently two schools of thought: 1) They function as gradient morphogens whereby similar cells respond in distinct ways to different concentrations of the same signal (reviewed by Wolpert 1989, Cooke 1995). 2) They act as short range inducers initiating sequential secondary signals or relay systems (Zecca et al 1995). Much of the current data available supports the second hypothesis, however, recent papers by Nellen et al (1996) and Lecuit et al (1996) identified a gene, (decapentaplegic - dpp gene) responsible for secreting a factor which exerts a long range influence on anterior and posterior compartments of the developing drosophilla wing. Furthermore, they show that different genes respond to different concentrations of the DPP protein - endorsing the morphogen gradient concept.

Retinoids represent a major class of non-peptide growth factor signals and have been implicated in several developmental and differentiation pathways including neural and mesodermal tissue differentiation (Strickland & Mahdavi 1973, Glass et al 1990, Helms et al 1996). The developing chick limb has been extensively used as an experimental system for studying retinoic acid (RA) action on gene expression. (see

Jessel & Melton 1992 for references). Much work has focused on events that occur at stage 18 and beyond when a distinct limb bud is present (Tabin 1995). However, limb buds are specified as early as stages 8 and 11 in chick embryos and appear in response to signals secreted from the lateral plate mesoderm (Cohn et al 1995 and Mahmood et al 1995). Two regions of the limb primordium are essential for the outgrowth and subsequent patterning of the chick limb bud, the zone of polarising activity (ZPA) and the apical ectodermal ridge (AER). It has recently been discovered that retinoic acid is a primary signal required for the establishment of the ZPA in these early stages of limb development (Helms et al 1996).

The ECM is known to be involved in the control of the diffusion of secreted factors throughout the developing organism. ECM components may reversibly bind soluble molecules and in doing so control their rate of migration through the cell population. Alternatively, ECM components such as proteoglycans may permanently bind secreted proteins as a means of localising high concentrations of specific factors such as FGF and TGFβ (Klagsburn & Baird 1991). The difficulty in detecting secreted Wnt proteins is thought to be due to rapid binding to heparan sulphate proteoglycans, (Hinck et al 1994). Binding to the ECM may also enhance or even activate several of these growth factors, indeed the association of FGFβ with ECM proteoglycans stimulates muscle cell differentiation (Rapraeger et al 1991).

There are a myriad of secreted factors which have been identified, many of which are intimately involved in gene regulatory activity which is manifest in a complex array of growth and differentiation control pathways. Many secreted factors are also involved in the regulation of other cell-cell interaction mechanisms and have been discussed further in the summary of this section 1.2.7.

1.2.3. Juxtacrine interactions.

Interactions between certain membrane bound proteins on apposing cell membranes can give rise to both adhesive and signalling events. Such processes have been termed juxtacrine signalling (Massague 1990). Although in many instances the membrane-bound factors can be proteolytically converted into soluble active forms, the cleavage represents conversion from one active form to another. Several juxtacrine molecules have now been identified and include: TGF-α, EGF, TNF-α, CD-21 (for references see Massague 1993). A well studied example is that of development of the R7 precursor cell to the R7 neuron in the compound eye of *Drosophilla* which occurs after an inductive interaction with the photoreceptor R8 (Tomlinson & Ready 1986). Adhesive interactions between the R8 ligand, (Boss) and the R7 receptor, (sevenless) was demonstrated by the heterotypic aggregation of only cells expressing those structures (Kramer et al 1991).

Data from *Drosophila, C. elegans* and vertebrates suggest that intercellular signalling by the Notch pathway plays a pivotal role in the differentiation of uncommitted

cells. The Notch / LIN-12 / GIP-1 receptor family have been implicated in a general mechanism of local cell signalling which mediates the specification of numerous cell fates during development (Heitzler & Simpson 1991, and reviewed in Tsakonas et al 1995). Notch of *Drosophila* is a 300kDa transmembrane receptor protein with 36 extracellular EGF-like tandem repeats. The putative ligands for Notch, which are membrane anchored and include *serrate* and *delta* also contain variable numbers of EGF-like repeats. Cell aggregation assays show that *delta* and *serrate* bind to the extracellular EGF regions of Notch, which is subsequently activated upon binding. Genetic and molecular analysis have revealed that Notch signalling events do not involve the transmission of specific developmental signals, but modulate the ability of cells to respond to such signals.

In general, cell adhesion systems are responsible for maintaining tissue integrity and overall tissue architecture. It has also been suggested that classical adhesion molecules, which link the cytoskeleten of apposing cell membranes and their associated proteins, couple physical adhesion to signalling events during morphogenesis. Because of their potential importance in influencing cell phenotype they are discussed in their own right in section 1.2.5.

1.2.4. Extracellular matrix.

The ECM is an important component of the extracellular environment. It is composed of proteoglycans, glycoproteins and glycosaminoglycans. ECM is present at the 2-cell stage of mammalian embryos and is a component of the environment of all cell types. *In vivo* cells attach to the basement membrane (a distinct sheath of ECM surrounding many tissue types) or a collagen rich interstitial matrix. The basement membrane is composed of two distinct layers; basal- and reticular-lamina. Several adhesive glycoproteins have been found within the basal lamina including collagen IV, laminin and fibronectin (Mosher et al 1992). Reticular lamina, produced by fibroblasts, is composed primarily of fibrillar collagens. In addition to offering structural support to cells it can form a physical barrier or selective filter to soluble factors and it has been shown to play a role in regulating the differentiated phenotype of cells.

Evidence for the importance of ECM components in the normal development of organisms comes from mutations which affect ECM proteins and receptors and experimental perturbation studies. *Drosophila* development particularly, has provided a rich source of information. For example, mutations in laminin is lethal in late embryogenesis (Hortsch & Goodman 1991) whilst mutations in the *Scabrous* gene (whose gene product has significant homology to vertebrate fibrinogen) results in altered spacing patterns of R8 photoreceptor cells (Baker et al 1990). In vertebrates many genetic and autoimmune diseases are associated with defective ECM interactions (Olsen 1995). For example, blistering diseases of the skin, such as *Bullous pemphigoid,* result from a disruption of hemidesmosomes, (a cell-to-basement membrane junction linked to

cytokeratin intermediate filament network, the main adhesion receptor being integrin $\alpha 6\beta 4$ - see below).

In vertebrates fibronectin is produced when gastrulation begins and is abundant during morphogenetic migratory cell movements such as neural crest migration (Boucaut et al 1984). Gastrulation is inhibited when the blastocoel cavity is injected with anti-fibronectin antibodies. The examples provided so far are consistent with the ECM playing a structural role during development. However, there is now a substantial body of evidence to suggest that cell interactions with the ECM can regulate many aspects of cell behaviour including proliferation and differentiation. Certain ECM adhesion receptors on the surface of cells interact with cellular signal transduction apparatus.

The most prominent and to-date the best studied cell-to-ECM adhesion receptors are the integrins. Integrins are expressed by a wide variety of cell types and cells often express more than one type (Hynes 1992a). All integrins are $\alpha\beta$ heterodimers, each subunit varying in size between 120-180kDa and 90-110kDa respectively. There is increasing evidence that integrins are involved in the transfer of information into and out of cells (Hynes 1992a).

In culture integrin-mediated cell adhesion to the ECM results in the formation of specialised adhesion sites termed focal adhesions. Localised at these sites are both structural proteins such as α-actinin, talin and paxilin and signalling proteins including focal adhesion kinase (FAK), cSrc and PKC. For this reason these adhesion sites have been described as "adhesion-dependent signal transduction organelles" (Lo & Chen 1994). Integrins have no known intrinsic enzymatic activity and are thought to mediate signalling events via their ability to assemble cytoskeletal frameworks such as the focal adhesion sites (Turner & Burridge 1991, Reszka et al 1992, LaFlamme et al 1992). Integrin mediated adhesion has also been shown to trigger the activation of lipid second messenger pathways via the activation of tyrosine kinase-dependent phospholipase C-γ (Kanner et al 1993).

There are many examples where the ECM has been shown to affect gene expression (Adams & Watt 1993). In monocytes for example the ligation of $\beta 1$ integrins with anti-integrin monoclonal antibodies induces the same pattern of gene expression seen after complex adhesion with other ECM molecules such as fibronectin (Yurochko et al 1992). A further example of integrin-mediated gene regulation can be found in the production of metalloproteinases in synovial fibroblasts. $\alpha 5\beta 1$ integrin binds to a distinct domain on fibronectin, in doing so it activates the transcription of metalloproteinases. However, the signal is inhibited if an opposing $\alpha 4\beta 1$ integrin binds to fibronectin (Huhtala et al 1995).

Many normal cell types in culture require anchorage to underlying ECM in order to proliferate (a mechanism lost in many transformed cells). Integrins are thought to be involved in the regulation of adhesion-dependent cell proliferation. They are capable of activating mitogen-activated protein (MAP) kinases (Chen et al 1994), which in turn are

known to be involved in the regulation of cell proliferation (Davis 1993). Thus, activation of MAP kinase appears to be dependent upon integrin-mediated changes in cell adhesion and cell shape (Zhu & Assoian 1995). Integrin-mediated interactions allow cells to 'sense' their spatial relationship with neighbouring cells during stages of controlled morphogenesis, tissue growth and tissue repair.

The ECM plays an important role in several majors areas of tissue morphogenesis *in vivo*, including cell spreading, cell migration and epithelial compaction (Gumbiner 1995). In addition to regulating the structure and organisation of cellular architecture the ECM can transduce signals relating to growth and gene expression. It contributes to ensuring that cell differentiation takes place in the right place at the right time.

1.2.5. Cell-cell adhesion.

Cell-cell adhesion is required for the assembly, maintenance, phenotype and overall architecture of cells and tissues in addition to providing a mechanical framework to co-ordinate cell-cell interactions. Cell-cell adhesion is a complex process, the molecules involved are often multiprotein complexes and can be involved in adhesion and cell-cell signalling. The adhesion molecules and their associated proteins can be grouped in to 3 categories: **i.** transmembrane proteins, **ii.** extracellular proteins, many of which are ligands for the transmembrane proteins, **iii.** cytoplasmic plaque/peripheral membrane protein. Group i, the transmembrane proteins, include the following superfamilies: cadherins, integrins, immunoglobulins, selectins, proteoglycans (e.g. syndecans). Cadherin-based adhesion forms one of the most important cell-cell interactions. Through their homophilic binding properties cadherins mediate important events during morphogenesis including cell sorting, cell polarisation and tissue morphology. More recently it has been suggested that cadherins may be involved in intercellular signalling. This is thought to arise via associations with cytoplasmic plaque and cytoskeletal proteins such as the catenins. β-catenin for example, has been shown to be phosphorylated in response to growth factors and can in turn decrease cell-cell adhesion (Kinch et al 1995).

1.2.5.1. *Cadherin-mediated cell-cell adhesion.*

Cadherins and their role in the formation of cell-cell adherens junctions represent one of the best studied adhesion systems. The cadherins are a family of cell surface membrane proteins which mediate calcium dependent homophilic cell-cell adhesion, they localise at points of cell-cell contact and are a principle component of adherens junctions or belt desmosomes (Hirano et al 1987) and are also thought to be involved in the formation of cell-matrix interactions (Geiger et al 1992). Two major sub-families have been identified: the classical cadherins which include, among others, E (epithelial), N (neuronal) and P (placental) cadherin (Takeichi 1988 & 1990) and desmosomal cadherins such as desmogleins and desmocollins (Buxton & Magee 1992). The latter

share conserved extracellular sequences with, and an overall structural similarity to the cadherins (Magee & Buxton 1991). Classical cadherins possess similar structural and functional domains, including sites for calcium binding, adhesive recognition, cytoskeletal interactions and post-translation phosphorylation sites. These proteins are believed to be responsible for the sorting phenomenon observed in culture, whereby cells expressing different cadherins segregate into homotypic populations (Nose et al 1988, Miyantani et al 1989). They are also responsible for mediating epithelial cell polarity (McNeill et al 1990, Watabe et al 1994, Marrs et al 1995). This process is fundamental to the development of a variety of tissues and organs (Rodriguez & Nelson 1989) and involves the redistribution of several important membrane proteins such as Na^+K^+-ATPase, to specific areas of the cell membrane (in this case basal-lateral).

It is generally believed that for functional adhesion to occur the cadherins must form associations with the intracellular cytoskeletal proteins - catenins (Gumbiner 1993), which themselves transduce signals received from the adhesion molecules. α-catenin links cadherins to the actin filament network (Rimm et al 1995). In PC9 lung carcinoma cells E cadherin is expressed but remains non-functional due to a lack of α-catenin expression (Watabe et al 1994). The re-expression of α-catenin induces a polarised cell phenotype and cell-cell association via the formation of cell-cell junctions (tight and adherens). Furthermore, the growth of the PC9 cells is retarded indicating a role for E cadherin-catenin associated growth control. β-catenin is believed to bind α-catenin to cadherin and is known to be involved in signal transduction (Kinch et al 1995). Post-translational disruption of the cadherin/catenin complex (rather than decreased expression levels) has been shown to be important in the process of dedifferentiation during epithelial transformation by the oncogene v-src (Behrens et al 1993); the transformed cells were subsequently rendered highly invasive. The effects of v-src were found to be associated with increased tyrosine phosphorylation of the β-catenin protein.

The broad function of cadherins in addition to their adhesive role was demonstrated by Hermiston et al (1995). Using embryonic stem cells stabley transfected with a dominant negative N cadherin mutant (NCADΔ), they were able to show that transfected cells poorly adhered to each other and to the ECM, their polarised phenotype was lost and they tended to apoptose with high frequency.

The development of the nervous system, in particular the neural crest, provides a useful system to study the role of adhesion in key morphogenetic events. Neural crest development begins with the dispersion, segregation and migration of cells. Following migration, the cells attach in various locations of the embryo and differentiate into neurons and the peripheral nervous system (Erickson and Perris 1993). When dispersion and migration begins N cadherin and N-CAM surface expression is lost (Thiery et al 1982) and cells show preferential associations with the ECM (Duband et al 1985). Following migration and reassociation into peripheral ganglia, the process is sequentially reversed and adhesion molecules such as N cadherin and a range of 'novel' cadherins are

expressed (Nakagawa & Takeichi 1995). The cadherin junctions which form are finely controlled by the co-ordinated activity of cellular kinases and phosphatases (Gavelle & Duband 1995)

It is clear that adhesion molecules do more than simply hold tissues and cells together; they provide a mechanism for integrating physical aspects of tissue morphogenesis with cell growth and differentiation. This role is reflected by the loss of adhesion proteins in many tumour cells and subsequent anchorage independent growth in culture and invasive potential *in vivo* (Behrens et al 1993, Birchmeier et al 1993). However, the re-expression of cadherin alone in such cells is often not sufficient to alter the tumorigenic phenotype of these cells (Navarro et al 1993) and this highlights the importance of integrated interactions with cytoskeletal proteins.

Several other types of cell-cell adhesion junctions are known to exist. They include desmosomes, found in epithelia and cardiac muscle which provide the mechanical strength required for the maintenance of tissue integrity and occluding junctions, i.e. tight junctions, which serve to regulate the permeability of the paracellular space between adjacent cells (Gumbiner 1987). Cadherins are also capable of regulating the formation of gap junctions and this is discussed in section 1.3.4.5.

1.2.6. Gap junctional communication.

Gap junctions occur in metazoan organisms, they are present at all times of development and lost only in some fully differentiated cells. They provide a potential mechanism for the distribution of developmental, positional and growth regulatory signals, though whether they perform such a role is still under investigation. Gap junctions are transmembrane channels which permit the passive transfer of cytoplasmic molecules, less than 1kDa in size throughout a coupled cell population. These molecules include ions and second messengers such as cAMP and Ca^{++}. As a result, they engender a cytoplasmic continuity (with regard to molecules less than 1kDa in size) within a coupled population. This continuity results in the establishment of a homeostatic pressure which is thought to be responsible for maintaining a homogenous cellular phenotype. Communication compartments (cells of the same compartment can communicate with each other but not with cells of other compartments) have been observed during embryogenesis and tissue development (Lo & Gilula 1979a, 1979b, Lee et al 1987, Nishi et al 1991 Kam & Hodgins 1992). Separation into sub-compartments is thought to be a pre-requisite for cells to express new phenotypes.

A developing tumour (essentially a differentiating, emerging cell population with an aberrant growth phenotype) must escape the growth control of the surrounding normal cells. Many published reports have recorded a loss of heterologous communication (communication between two different cell types or cell lines) between normal and transformed cells *in vitro* (Mehta & Loewenstein 1991, Kalami et al 1992,

Mikalsen 1993). A review of the evidence for the role of GJIC in growth, development and carcinogenesis is provided in section 1.4.5.

1.2.7. Summary of the cell-cell interactions involved in the determination and maintenance of cell phenotypes.

The various interactions described appear to play pivotal roles in the initiation and maintenance of cell differentiation. Needless to say the various mechanisms of cell-cell interactions outlined above do not operate independently. Cell differentiation and regulation of morphogenesis require, among other things, complex interactions between adhesion receptors, the cytoskeleton and networks of signalling pathways. Signalling pathways generated by integrin-mediated adhesion (and possibly cell-cell adhesion dependent signalling) are themselves influenced by extrinsic signals arising from classical growth factors. Gap junctions provide the apparatus for intercellular internal signalling pathways. These may be important for determining and maintaining the differentiated state either by suppressing individual phenotypes as a result of homeostatic pressure or by direct signalling molecules (as yet unidentified). GJIC is known to be regulated by cell-cell adhesion systems (Musil et al 1990, Hertig 1996), and modulated in response to various soluble factors such as the Wnt's and ECM components including proteoglycans and glycosaminoglycans (see section 1.3.4).

Cell adhesion interactions can be regulated by long-range signalling pathways, for example, β-catenin is thought to be a component of the Wnt signalling pathway in *Xenopus* embryos and may contribute to embryonic patterning (Peifer 1995). In mammalian cell culture systems Wnt-1 expression results in the accumulation of β-catenin and its associated protein - plakoglobin, leading to widespread and stronger cell-cell adhesion. Such interactions offer a potential mechanism for the specification of cell type boundaries (Hinck et al 1994).

Tissues are dynamic structures, chemical signals and cell-cell interactions result in altered gene expression and structural changes to cell morphology. A unifying theory of cell migration, proliferation and tissue development has been proposed. It is based on cellular changes that result from alterations in the extended cytoskeleton, (a continuous structural support network spanning from a cell's nucleus through its cytoskeleton, across its associated ECM via intercellular junctions and on into neighbouring cells). The end result is a physical integration of all cells and nuclei within tissues (Wang 1993, Ingber 1994). The role of mechanical forces generated by cell-cell interactions in tissue development is a growing field and is termed *cellular tensegrity* a full review can be found in Ingber et al (1994).

It has been shown in this section 1.3.4 that the cell phenotype is determined not only by the genes which a particular cell expresses but also by the environment in which it resides and the interactions it makes with other cells. In the following section the role of GJIC in growth and development of organisms is explored further.

1.3. Gap Junctional Intercellular Communication.

The evidence which supports a role for GJIC in development and cell growth control is mainly circumstantial - see section 1.4.5. The homeostatic pressure which arises due to communication within a coupled cell population would appear to suppress the expression of individual cell phenotypes. In order for cells to express new phenotypes they must first escape this growth control by forming new sub-compartments. Gap junctions are known to perform other roles, in excitable tissues for example. At electrical synapses GJIC facilitates rapid propagation of ionic current (Jaslove & Brink 1986), thus providing the means to regulate neuronal assemblies and the ionic environment in which neuronal activities occur (DeVries & Schwartz 1989). Within this section gap junction-mediated cell-cell interactions will be discussed and their role in growth control and the maintenance of cellular phenotypes investigated. However, some basic information on gap junction structure, formation and subsequent permeability is required, this is provided below.

1.3.1. Gap junction structure.

The gap junction channel is composed of two hemi-channels termed connexons. Each hemi-channel joins end-to-end with another, provided by the apposing membrane of another cell. Each connexon is composed of 6 sub-units, (each subunit is composed of 4-α helices), arranged symmetrically around an axial water-filled channel with a diameter of 1-2nm (Figure 3.1). There is controversy as to what protein is the principle channel component of the connexon (for review see Finbow & Pitts 1993). The debate revolves around two proteins, ductin and connexin (Cx).

1.3.1.1. *Ductin.*

Ductin is a 16kDa protein which is also a major component of vacuolar-ATPase proton channel (Mandel et al 1988) and, as such, is present in all cells regardless of whether they form gap junctions. It has been found in gap junction preparations from both vertebrate and invertebrate sources (Finbow et al 1984 and 1993). Ductin is a highly conserved membrane protein. Sequences obtained from plants, fungi, vertebrates and invertebrates share ~80% sequence homology. There is also little difference in the size of ductin obtained from such diverse sources (150-160 residues; Finbow et al 1992). Ductin is composed of 4 hydrophobic segments joined by short hydrophilic segments. In the connexon, each of the 4 hydrophobic segments are thought to traverse the membrane as an α-helix (Holzenburg et al 1993). Antibodies to invertebrate ductin bind to isolated gap junctions (Buultjens et al 1988) and gap junctional regions in tissue sections (Leitch & Finbow 1990). GJIC has also been inhibited by the injection of anti-ductin antibodies into mammalian and invertebrate cells (Finbow et al 1993). These data (for a more extensive review see Finbow & Pitts 1993) show ductin to be involved in gap junctional

communication and together with the structural studies (Holzenburg et al 1993, Girvin & Fillingham 1994), make ductin a suitable and likely component of the gap junction channel.

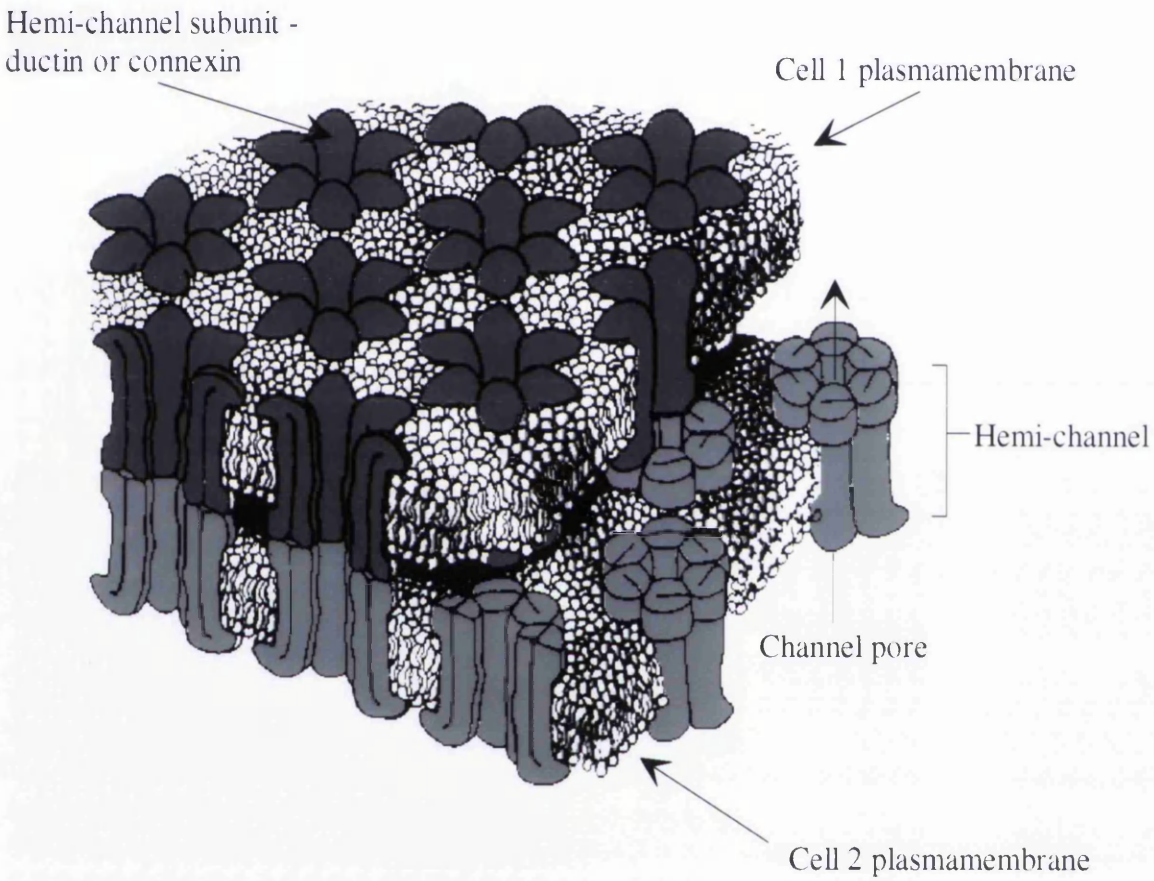
1.3.1.2. *Connexins*.

Connexins are a family of proteins ranging in size from 26kDa-50kDa and are expressed in a cell specific manner in a variety of mammalian cells. Twelve members of the murine connexin gene family have been described (Willecke et al 1991) and they are expressed in one or more cell type and often more than one connexin is expressed (Dermietzel & Spray 1993). Although it is argued that these proteins form the gap junction channel no connexins have been identified in invertebrates despite the successful isolation of gap junctions from such organisms (Finbow et al 1984). In addition, functional analogies appear to exist between gap junctions and plant plasmadesmata, however, despite concerted efforts (Xu et al 1990, Yaholm et al 1991) no connexin homolog has been discovered (Mushegian & Koonin 1993).

There is currently little structural data available for these proteins and that which is, does not satisfactorily relate to the structure of the gap junction connexon as imaged by X-ray diffraction and AFM studies (Makowski 1988, Hoh et al 1991, Yeager & Nicholson 1996). The different connexins show homology over the N-terminal region (~20kDa) but show considerable variation in length and sequence over the C-terminal domain (Kumar & Gilula 1992). It has been proposed that the 4 hydrophobic domains of the N-terminal region span the membrane as α-helices. Although this fits in with the proposed connexon structure the large variable C-terminal domains can not be accounted for. It has been suggested that this region is "dissorded" and therefore can not be visualised but it seems unlikely that the extra mass which the C-terminal region accounts for (60% of Cx32 and 110% for Cx43) remains undetected even when analysed by various imaging techniques (Tibbets et al 1990, Sosinsky 1992).

Because many studies of connexins are based upon the supposition that they form the gap junction channel there has been little progress into the potential array of alternative functions which this large family of proteins may have. It has been shown that the formation and control of gap junctional communication is highly complex and is likely to be under the control of multiple gene products (Macdonald 1985). A wide variety of cell-cell interactions are known to influence GJIC (see section 1.3.4) and connexins may be the target of such diverse signalling pathways. Furthermore, they may be involved in generating the patterns of communication observed *in vivo* (e.g. Pitts et al 1986), which change spatially and temporally during different stages of development. The putative role of the connexins in the generation of gap junction specificity leading to the establishment of communication compartments *in-vivo* and in tissue culture is discussed in section 1.4.5.

Figure 3.1. Schematic representation of the proposed gap junction connexon



Based on a diagram by Makowski et al 1977.

1.3.2. The formation of gap junctions.

Until the issue regarding the structure of the channel is settled there can be little progress made on how the gap junction forms. However, it is known that when gap junctions form they do so rapidly (within minutes) when cells are brought into close contact with each other (Rook et al 1988, 1990). The fact that this can occur in the presence of protein synthesis inhibitors (Rook et al 1990) suggests that channel precursors exist within the cell or at its surface membrane. If this is so then interaction of the two hemi-channels may cause the channel to open. Alternatively, the channel may form as a closed structure which is opened by the interaction of connexins or other, as yet unidentified, gap junction associated proteins on apposing cell membranes. Once the two hemi-channels have docked the channel is subject to gating mechanisms which can rapidly switch the channel between open and closed states (section 1.3.4).

It has been shown that the hemi-channels protrude by only 1-2nm into the extracellular environment (Hoh et al 1991), therefore, the membranes of the apposing cells must be brought into very close apposition before functional channels can form. Cell-to cell adhesion molecules are the most likely candidates to bring about initial close apposition followed by other, as yet unidentified molecules, which bring the membranes even closer together. Connexins may also be involved in bringing two hemi-channels into close apposition. However, the absence of these proteins in invertebrates despite the presence of functional gap junctions would suggest that the connexins play a more regulatory role in vertebrates and add further complexity to the gap junction structure not recognised by current models of the connexon.

1.3.3. Gap junction permeability.

Gap junction channels permit the passive diffusion, between cells, of molecules less than ~1kDa. This includes, ions such as Ca^{++}, small cytoplasmic molecules including second messengers and metabolites such as nucleotides, (Pitts & Simms 1977, Neyton & Trautmann 1985, Saez et al 1989,) but excludes macromolecules such as nucleic acid, polysaccharides and large proteins. This metabolic co-operation can support metabolic activity within cells of a coupled population, when some of which, for some reason, lack a full range of such activities (Pitts 1980). Metabolic co-operation has been readily demonstrated in tissue culture systems; for example, cells deficient in hypoxanthine phosphoribosyl transferase ($HPRT^-$) are unable to make purine nucleotides from hypoxathine and cells which lack thymidine kinase (TK) are unable to make thymidine nucleotides from thymidine. When these two cell types are co-cultured in the selective medium, HAT, survival and growth is dependent on the mutual exchange of nucleotides and occurs only when the cells form heterologous gap junctions (Pitts 1971, Pitts & Simms 1977). Newly developing cancer gene therapies based on enzyme pro-drug systems exploit metabolic co-operation (in this case the transfer of toxic metabolites) as

a means of spreading the toxicity to cells not modified by the therapeutic gene (Bi et al 1993).

Gap junctions mediate electrical coupling within excitable tissues. In heart for example gap junctions mediate the propagation of the action potential resulting in a co-ordinated muscle contraction (Fawcett & McNutt 1969). Gap junctions are known to be permeable to several molecules involved in the growth and regulation of cells such as, cAMP, IP_3 and Ca^{++}. These findings have led to the proposal that GJIC is involved in cell differentiation in normal developmental pathways, see section 1.4.5.

There are essentially three methods used to measure gap junctional communication in culture. The first and most widely used is the microinjection of fluorescent dye (usually Lucifer yellow CH, with a MW of 440 and molecular radius of 0.7nm). A single cell within a monolayer is injected with the dye for a given time. If the population of cells is connected by gap junctions the dye diffuses readily into adjacent cells, demonstrating 'dye coupling'. Another type of assay demonstrates coupling by the transfer of labelled metabolites (and subsequent analysis by autoradiography). The assay is termed nucleotide transfer and was first used by Pitts & Simms (1977). This technique has biological relevance because it is concerned with the transfer of metabolites and is particularly amenable to the detection of gap junctional communication between cells of different types - heterologous communication. The third assay commonly used is the measurement of gap junctional conductance, where the extent of electrical coupling between cells is measured. This technique is often used in conjunction with the paired *Xenopus* oocyte system. This system permits direct measurements of cell-cell communication to be made and related to expression of specific connexins (Swenson et al 1989).

GJIC has been measured in intact tissues using the dye injection assay. After injection the tissue is fixed, sectioned and dye spreads visualised. Analysis of dye spreads in various tissues including skin and in mouse embryos led to the discovery of communication compartments (Pitts et al 1986, Kalimi & Lo 1988, see section 1.4.5.1).

1.3.4. The regulation of GJIC.

If GJIC is involved in the growth regulation and development of multicellular organisms then control of communication levels is likely to be critical. Gap junctional regulation may also be necessary during states of disease, injury or exposure to toxins. In such instances it is thought likely that normal undamaged cells would wish to isolate themselves from damaged ones to prevent the leakage of small cytoplasmic molecules. Gap junctional communication can be modulated at four levels:

1). Gating. This refers to the modification of channels whilst in the junction and involves switching between an open and closed conformational state. This is likely to be a fast means of control.

2). Modification of gap junction proteins during synthesis and transport to the plasmamembrane. This is likely to include phosphorylation and subsequent conformational changes of gap junction proteins.
3). Turnover. Evidence of such a regulatory mechanism is limited and rests on the observations of connexin-antibody-reactive material present in the vesicles proximate to the plasma membrane (Atkinson et al 1995), in addition to the relatively short half-life (5hrs) of connexins Cx26, Cx32 & Cx43 (Traub et al 1989, Laird et al 1991). Such short turnover times are rapid enough to regulate levels of gap junctions by control of synthesis.
4). Gene regulatory mechanisms. Many studies on GJIC regulation have concentrated on the effects of various modulators (see below). Therefore little has been discovered on how gap junction genes are regulated. Further genetic analysis may lead to a better understanding of the tissue specific expression of certain gap junction associated proteins such as the connexins (for further discussion see Kumar & Gilula 1992).

Rapid and often transient modulation of GJIC is thought to occur by junctional gating (Spray et al 1981, Giaume et al 1989, Veenstra 1990). Two models of gating mechanisms have been proposed. Firstly, one in which the cytoplasmic end of the channel is capped by some form of protein structure (Makowski et al 1984) and secondly one in which the connexon subunits twist along the channel axis, to open/close the channel - an action similar to that of the camera lens aperture (Unwin & Ennis 1984).

Junctional conductance and single channel conductance measurements (see Veenstra & Brink 1992 for methodology and discussion of technique) permit the proportion of open gap junctions to be estimated. The measurements vary depending on the sample, for example, ~1% of channels are open in the endings of Mauthner cell synapses (Lin & Faber 1988) whereas in the crayfish septate axon all junctions appear to be open in their native state (Zamphigi et al 1988). It has also been shown that individual channels can switch between open and closed conformations within milliseconds (DeHann 1988). Further structural analysis of the channel protein and connexon complex may provide further insight into how gating mechanisms may work.

Changes in junctional permeability or conductance can be induced by a variety of different agents, those thought to be of particular importance are listed below, however, in many instances the actual mechanisms by which they exert their effect are poorly understood.

1.3.4.1. *Cyclic AMP.*

The signalling molecule cAMP is known to be involved in the control of gap junctional permeability (Flagg-Newton et al 1981a & b, Saez et al 1986, Mehta et al 1992, Atkinson et al 1995). Cells generally respond to cAMP by increasing their levels of gap junctional communication (Saez et al 1986), there are however exceptions such as

horizontal cells from fish retina, where a decrease is recorded (Teranishi et al 1983). Atkinson et al (1995) showed that an increase in communication occurred within 30 minutes of continuous exposure to cAMP. Levels increased by four fold after a further 24 hours.

The upregulation of GJIC by cAMP appears to operate via more than one mechanism. For example several reports have shown that cAMP - upregulated gap junctional communication is sensitive to protein synthesis inhibitors (Traub et al 1987) and that connexin mRNA levels increase in parallel to the increased communication (Mehta et al 1992, Schiller et al 1992). Atinkinson et al (1995) has confirmed that an increase in the junctional permeability of mouse mammary tumour cells is associated with an increase in the number of gap junctions, as shown by freeze-fracture EM. However, it was found that Cx43 mRNA and Cx43 protein levels did not change. This would suggest a that the formation of further channels is at the expense of the pool of Cx43 elsewhere within the cell.

The stimulation of cAMP-dependent protein kinase A (PKA) in response to elevated levels of cAMP (Taylor et al 1990), activates the transcription factor - CREB, (cAMP response element-binding factor), causing increased transcription of cAMP responsive genes. Sequences similar to the cAMP response element (CRE) are located near the transcription start site of Cx32 gene (Miller et al 1988). This may account for the increased expression of this protein observed by Traub et al (1987) when embryonic hepatocytes were stimulated with cAMP.

In several instances artificially increased levels of cAMP has resulted in a reduction in cell proliferation (Cho-Chung 1985). Thus, if gap junctions are involved in the spread of growth regulatory signals then the effect of cAMP may be to increase both the amount of regulatory molecules and the mechanism by which they can spread - a convergence of cellular signalling pathways.

1.3.4.2. *Calcium.*

Ca^{++} is an important second messenger. It is released in to the cytosol after receptor-mediated extracellular signalling induces the IP_3 signalling pathway. Ca^{++} is then bound by calmodulin which binds to other proteins thereby altering their activity. Transmission of Ca^{++} via gap junctions has been implicated in signalling neuronal death during development (Wolszon 1994a - see section 1.4.5).

Ca^{++} has been reported to downregulate GJIC (Spray et al 1982). However, the concentration required is atypically high for most Ca^{++} effects on normal cells. Such high Ca^{++} levels may only be achieved after injury when it may provide an important means of isolating damaged tissue from surrounding healthy cells.

There has been a prolonged argument regarding the role of Ca^{++} versus that of H^+ in the regulation of GJIC. Turin & Warner (1977) showed that lowering pH in *Xenopus* embryo cells led to the down-regulation of junctional communication.

However, in 1978 Rose & Rick showed that Ca^{++} levels were elevated in response to either increasing or decreasing pH and junctional communication was subsequently decreased.

1.3.4.3. *Phorbol esters.*

Tumour promoting phorbol esters such TPA appear to act as variable modulators of GJIC. Many cell lines show a downregulation of GJIC in response to TPA treatment, (for review see Klaunig & Ruch 1990). However, TPA has also been shown to increase dye transfer across the dermal-epidermal boundary when applied to intact mouse skin (Kam & Pitts 1988) and electrical coupling is also increased in some systems including cardiac muscle (Spray & Burt 1990).

TPA is thought to inhibit gap junctional communication through activation of protein kinase C and subsequent hyperphosphorylation of Cx43 (Brissette et al 1991, Gainer et el 1985). There are 23 serine sites within Cx43 which may potentially be phosphorylated. However, the effects of PKC activation are widespread and complex. The cascade of signalling changes which can occur include the activation MAP kinase (Nori et al 1992). Studies by Matesic et al (1994) showed that the rapid reduction in gap junctional communication, (within 5min) in rat liver epithelial cells was due to Cx43 hyperphosphorylation (this was manifest in the occurrence of a higher molecular weight band during Western blotting analysis) and a concomitant reduction in the number of Cx43 immunostained gap junction plaques. However, the correlation between Cx43 phosphorylation and decreased communication is in contrast to that of Musil et al (1990) who correlated connexin phosphorylation with increased gap junctional communication. These differences are possibly due to the extent and sites of phosphorylation within the protein.

However, the fact that chemicals such as TPA are non-specific and cause a cascade of potentially important phosphorylation events in other signalling pathways (Gosh et al 1990, Nori et al 1992) mean that it is not possible to draw causal relationships between changes in GJIC and changes in cell physiology. TPA-mediated changes in GJIC during multistage carcinogenesis are considered in section 1.4.6.

1.3.4.4. *Retinoids.*

Retinoids are naturally occurring and synthetic compounds derived from retinol (vitamin A_1). They are effective modulators of proliferation and differentiation of keratinocytes *in-vivo* and *in-vitro* and have profound yet diverse effects on the patterning of skeletal elements in various vertebrates (Maden 1982 & 1983, Tickle 1983). Nuclear receptors for retinoids (RAR), which modulate gene transcription in the regulatory regions of retinoid-responsive genes, have been discovered (Glass et al 1990) and may be responsible for the diverse range of actions on gene expression. Retinoids have also been used as cancer preventative agents in epithelial tissue. For example, the recurrence rate

of patients at high risk from second primary malignancies of the head, neck or lung decreases after systematic retinoid treatment (Hong et al 1990)

The effects of retinoids on GJIC vary depending on cell type, retinoid type and concentration (Pitts et al 1986, Yamasaki & Katoh 1988, Mehta & Loewenstein 1991). For example, gap junctional communication is blocked in animal cells at high concentrations (10^{-4}M; Pitts et al 1986) and blocked in cultures of mouse C3H10T1/2 cells at low concentration (10^{-10}M; Mehta et al 1989). At physiological concentrations (10^{-8}M) the homologous communication of C3H10T1/2 cells is increased, but their heterologous communication with chemically transformed relatives is decreased (Mehta & Loewenstein 1991). The expression of Cx43 was found to be regulated by retinoic acid in intact human skin (Guo et al 1992).

1.3.4.5. *Adhesion mediated regulation of GJIC.*

There is abundant evidence to suggest that cell-cell adhesion can control GJIC (Mege et al 1988, Keane et al 1988, Jongen et al 1991, Meyer et al 1992). Its role may simply be to bring cells into close apposition so that gap junctions can form. In a broader sense it may be involved together with cell-matrix interactions in ensuring that GJIC occurs in the correct cellular environment.

Cells expressing different cadherins sort out in mixed cultures indicating a role in selective cell-cell adhesion (Miyatani et al 1988 and Nose et al 1988). This adhesive specificity is thought to be involved in the specificity of gap junction formation observed in culture and *in vivo* (see section 1.4.5.2.b). Mege et al (1988), have shown that transfection of poorly coupled S180 cells with a cDNA for E-cadherin results in increased adherens and gap junction formation. This was observed by ultrastructural and dye transfer analysis. The frequency of expression of both types of junction was sharply decreased by treatment with anti-E cadherin Fab' antibodies.

Similarly, Meyer et al (1992) showed that the formation of gap junctions was dependent upon the formation of N cadherin-mediated adherens junctions. Using antibody Fab' fragments against either N cadherin or the 1st and 2nd extracellular domains of either Cx32 and Cx43, they were able to disrupt the reaggregation of Novicoff hepatoma cells. This was associated with a loss of dye coupling and EM analysis confirmed the loss of adherens and gap junctions. These experiments demonstrate the importance of close apposition between cells for the formation of gap junctions and suggest a possible role for connexins in cell-cell adhesion. Further experiments are required to determine whether adhesion-mediated signalling events are also important for junction formation.

Studies by Mege et al (1988), Musil et al (1990) and Meyer et al (1992) indicate that cadherin-based adhesion contributes to the establishment of gap junctional communication. It has been suggested that gap junction-mediated signal transduction modulates cell adhesion during compaction of the preimplantation mouse embryo (see

Lee et al 1987 and references there-in). A recent study by Paul et al (1995) utilised a dominant-negative chimeric Cx32/43 protein to block gap junctional communication. Following injection of chimeric mRNA into anterodorsal blastomeres of 8-cell stage mouse embryos, a loss of gap junctional communication and concomitant delamination of subsequent surface ectoderm occurred. Although the results were similar to those observed by Levine et al (1994) and Heasman et al (1994) in which cadherin based adhesion in early *Xenopus* embryos was perturbed by the expression of a cadherin dominant-negative mutant, they differ to the results of the connexin-antibody perturbation studies by Warner 1984, Lee et al 1987 and Fraser et al 1987, who found that adhesion was not perturbed. However, Paul et al (1995) were unable to rescue embryos expressing the chimeric connexin by co-injection of E-cadherin mRNA. The results are complex and may reflect the interplay between adhesion and GJIC. However, the possibility that connexins may be forming adherent structures was not considered as current dogma suggests that connexins only play a role in gap junction formation.

The post-translational phosphorylation of Cx43 appears to be a general phenomenon in communication efficient cell lines (Musil et al 1990, Crow et al 1990, Lau et al 1991 and Meyer et al 1992). Musil et al (1990) showed that poorly coupled mouse sarcoma cells (S180) express two species of Cx43. The unphosphorylated species, designated Cx43-NP (43kDa), predominates over the serine phosphorylated Cx43-P_1/P_2 (45-47kDa) species. However, after transfection of the cells with E cadherin cDNA and subsequent expression of the protein, the phosphorylation pattern of the connexins is reversed. The morphology of the cells changes to one of a more epitheloid type and dye coupling significantly increases. It has been postulated that phosphorylation of Cx43 (though not hyperphosphorylation) may be important in the formation and maintenance of functional gap junctions (Musil & Goodenough 1991).

1.3.4.6. *Other effectors of GJIC.*

Junctional permeability measured electrically can be influenced by a range of other agents including, anaesthetics (Niggli et al 1989), fatty acids (Giaume et al 1989), growth factors e.g. EGF (Kanemitsu et al 1993), TGF-β (Maduhkar et al 1989) and secreted molecules e.g.Wnt-1 and activin B (Olson & Moon 1992). In this latter study it was shown that polarity in gap junctional communication existed in 32-cell stage *Xenopus* embryos, in that dorsal cells are relatively more coupled than ventral cells. The ability of factors, which are known to induce mesoderm induction, to increase GJIC were examined. Expression of Wnt-1, activin B and TGF-β increased communication in ventral cells, however, the other mesoderm inducing factor - FGF did not upregulate gap junctional communication. Furthermore, subsequent defects in the dorsoventral axis correlated to increased junctional communication, although this does not provide direct evidence that junctional communication is involved in the establishment and maintenance of this axis. Studies by Olsen et al (1991) showed that misexpression of Wnt-8 led to

axial duplication in *Xenopus* which in turn was correlated to increased ventral GJIC. Developmental defects were also observed when activin B was misexpressed in *Xenopus* embryos (Thomsen et al 1990).

EGF, PDGF and TGF-β have all been shown to decrease levels of GJIC (Maldonado et al 1988, Madhukar et al 1989). EGF induced disruption is associated with the serine phosphorylation of Cx43. The cascade of events leading from EGF receptor activation and connexin phosphorylation are thought to be mediated by MAP-kinase (Kanemitsu 1993).

Interactions between cells and their associated ECM are believed to be important in regulation of GJIC. For example, levels of junctional communication in primary liver cultures were upregulated by the presence of proetoglycans and glycosaminoglycans in the medium (Spray et al 1987). These polysaccharide and protein-polysaccharide complexes are present in abundance in the ECM of liver cells. It is not yet known if integrin-mediated signalling plays any role in GJIC regulation.

Certain oncogenes, including src, ras and SV40 downregulate GJIC (Bignami et al 1988a, 1988b). These observations have contributed to the hypothesis that modulation of GJIC is an important step during multi-stage carcinogenesis and tumour development. This is reviewed in more detail in section 1.4.6.

1.4.5. The role of signalling via gap junctions in normal growth and development.

There are several lines of evidence to suggest that gap junctions are involved in the normal growth and development of multicellular organisms.

1). They are present in all complex multicellular organisms, particularly during early embryonic stages of development when cell-cell interactions leading to cell commitment and differentiation occur (Lo & Gilula 1979a).

2). Several growth regulatory and developmental factors can pass through gap junctions or modulate gap junctional communication (Spray et al 1982, Pitts et al 1986, Mehta & Loewenstein 1991, Atkinson et al 1995).

3). There is spatial and temporal alteration of the pathways of GJIC and gap junction associated proteins during growth, differentiation and development (Risek & Gilula 1991, Brissette et al 1994).

4). The establishment of communication compartments *in vivo*, (groups of cells coupled to cells of the same compartment but not with those of other compartments), is closely associated with the appearance of different cell lineage's occurring during development (e.g. Kam & Hodgins 1992).

5). GJIC is altered in different stages of carcinogenesis and the gap junction associated protein - Cx43 has been shown to restore communication and normalise the growth of transformed cells (Yamasaki 1991, Mehta et al 1991).

Gap junctions connect the cytoplasm of one cell to another and allow access to an array of potential signalling and growth control molecules. In theory, signals received by only a few cells that give rise to second messengers, may be relayed throughout a population of cells providing a co-ordinated response, which may result in that cell population following a new lineage pathway. Alternatively, a cell which may receive a potential inductive signal may be prevented from differentiating by the homeostatic pressure which gap junctions mediate.

In normal mouse embryos morphologically identifiable gap junctions first occur at 8 cell compaction stage (Ducibella et al 1975, Magnuson 1977). This correlates with the onset of electrical coupling between all blastomeres of 8-cell embryos (Lo & Gilula 1979). Cells can be taken from the 8 cell stage embryo and replaced on either the inside or outside of the embryo. Those on the outside become trophoblastic cells and those on the inside contribute to the inner cell mass (ICM). Beyond the 8 cell stage cells become determined (although not irreversibly). For example, if cells of the ICM are removed from late stage blastula and maintained *in vivo* some revert to trophoblastic cells, suggesting cellular phenotypes are influenced by environmental factors such as the presence of other determined cells. Several mechanisms may be responsible for the environmental influence which is excerted, including homeostatic pressure mediated by gap junctions.

In addition to these early embryonic observations gap junctional communication is believed to be involved in later stages of development and has recently been shown to be involved in neuronal growth cone development. In leech embryos developing anterior pagoda (AP) neurons transiently overlap and detect each others presence adjusting their branching pattern accordingly. Removal of an AP neuron by death or by mechanical means results in a nearby AP axon occupying the vacated area. Thus, it appears the neurons communicate there presence or death to other AP neurons (Wolszon 1994a & b). Wolszon et al (1994a) have shown, via dye injection and electrophysiology, that gap junctions are present between the transient, contacting axons. Furthermore, they identified calcium waves passing through gap junctions which appeared to signal neuron death (Wolszon et al 1994b). In humans, mutations in various connexin genes have been implicated in several genetically inherited diseases of the nervous system (Patel & Lupski 1994, Spray & Dermietzel 1995).

Connexin knockout mice are now beginning to be produced. Mice deficient in Cx43 (after targeted mutagenesis) survive to term (Reaume et al 1995). However, at birth the mice die as a result of a failure in pulmonary gas exchange caused by a blockage in the right ventricular outflow tract from the heart. Cx43 is clearly an important protein in foetal heart development, despite this its absence would appear to be compensated for.

1.4.5.1. *Communication compartments.*

The establishment of communication compartments during embryogenesis and early development and the association of such compartments with the emergence of new lineage's is further evidence that gap junctional communication is involved in growth regulatory and developmental processes (Lo & Gilula 1979a & b, Warner & Lawarence 1982, Kam et al 1987, Serras et al 1989, Yuste et al 1992). This has strengthened the hypothesis that communication compartments restrict an *individual* cells ability to form new phenotypes and permit *groups* of cells to differentiate and form new lineage's. Communication compartments have been observed throughout several stages of embryonic morphogenesis (Fraser & Byrant 1985, Kalimi & Lo 1988, Serras et al 1989, Dahl et al 1996) and in several tissues, including skin (Pitts et al 1986), hair follicles (Kam & Hodgins 1992), early chick limb (Allen et al 1990) and neuroepithelium (Martinez et al 1992). The putative mechanisms which generate such communication patterns are discussed in section 1.4.5.2.

The communication compartments in skin (Pitts et al 1986, Kam & Hodgins 1992) are, in general, associated with subpopulations of different differentiated cells. In the developing hair follicle, compartments were established between cells following different differentiation pathways. Kam & Hodgins (1992) found that dye spreads were extensive between cells of the hair bulb matrix but cell populations committed to the development of the inner root sheath were in separate compartments.

Gap junctional communication appears to be important in the patterning of chicken skin and feather development, (Serras et al 1993). Dye transfer studies revealed compartments in mesoderm and epidermis with preferential dye spreads along the anterioposterior axis of the feather placode and restricted spread at the feather bud/interbud boundary during early feather bud development.

In culture, restricted heterologous communication is observed between normal cells and many types of transformed cells. The situation is perhaps analogous to the establishment of a new phenotype (transformed in this case) and may offer a useful paradigm with which to study the process of gap junction specificity which may be responsible for the establishment of communication compartments *in vivo*.

1.4.5.2. *Gap junction specificity.*

The molecular basis of gap junctional specificity which leads to the establishment of communication compartments is not clearly understood. Expression studies using dominant negative mutants, chimeric proteins, and antibody perturbation experiments have enabled gap junction formation events to be dissected. The connexins and the cell adhesion molecules may mediate junctional specificity.

1.4.5.2.a. *Connexins and specificity.*

Because connexins are a large family of proteins and they show spatiotemperal expression during different stages of development (Paul 1985, Beyer et al 1989, Gimlich et al 1990, Risek & Gilula 1991, Goliger & Paul 1994) it has been suggested that they are involved in gap junction specificity.

Communication compartments are present in postimplantation mouse embryos (Dahl et al 1996). These compartments were defined by lucifer yellow dye spreads and were revealed in embryonic and extra-embryonic germ layers of ectoderm, mesoderm and endoderm. Expression studies and immunofluoresence analysis locate Cx43 exclusively in embryonic cells whilst Cx31 localises to extraembryonic cells and was maintained when the axial polarity of the mouse embryo was established. The results suggest that differential connexin expression is responsible for the establishment of these compartments.

However, Risek et al (1992) showed that Cx43 is expressed by different cell lineage's within the hair follicle despite the fact that different lineage's form separate communication compartments (Kam & Hodgins 1992). Connexin expression has also been analysed in developing rat skin and new born mouse epidermis (Risek et al 1991, Kamibayashi et al 1993). From the results it was concluded that multiple connexin genes contributed to epidermal and follicular morphogenesis and that communication compartments were established.

Gap junction specificity has also been observed in a number of *in vitro* cell systems (Pitts and Burk 1976, Prowse 1992). Using the *Xenopus* oocyte expression system it has been shown that expression of certain connexins can lead to both homotypic and heterotypic gap junction formation (Dahl et al 1987, Swenson et al 1989, Barrio et al 1991). However, different connexin combinations are restricted in their ability to induce junction formation. White et al (1995) found that functional channels formed only when Cx40 and Cx37 were expressed. Conversely, junctions formed when Cx46 was expressed together with Cx - 26, 32, 37, 43 and 50. This has been supported by the studies of Bruzzone et al (1993) who showed that *Xenopus* oocytes expressing Cx40 were able to form junctions with oocytes expressing Cx37, however no communication was not detected between oocytes expressing Cx40 and Cx43. The lack of gap junction formation after the heterotypic expression Cx40 and Cx43 may be of physiological relevance: within the vascular wall of Rats Cx40 is expressed in endothelial cells, whereas in adjacent smooth muscle cells Cx43 is expressed. The purpose of this differential expression may be to limit communication between the two cell types. This is supported by the finding *in vivo* that no communication is detected between the two cell types (Segal & Beny 1992). Specificity of gap junction formation has also been observed in connexin transfectants in culture (Tomasetto et al 1993).

Using pairs of *Xenopus* oocytes to express chimeric connexin constructs it has been shown that heterotypic expression of connexins leads to the formation of gap

junctions with different gating properties (Bruzzone 1994). In addition to their possible role in gap junction specificity, connexins have been implicated in the regulation of channel size (Steinberg 1994, Elfgang et al 1995). However, the evidence for this is limited and the majority of reports show that channel size is fixed, when open, at ~1-2nm.

1.4.5.2.b. ***Cadherins and specificity.***

It has already been shown in section 1.3.4.5 that GJIC has a close association with cell-cell adhesion and that cell-cell recognition mediated by the cadherins may well be a pre-requisite for the formation of gap junctions (Mege et al 1988, Jungian et al 1991). This is not surprising from a formation perspective, given that gap junction connexons protrude by only ~1nm and therefore the membranes need to be brought into close apposition to allow the formation of functional channels. This is consistent with the finding that antibodies to N-cadherin disrupts gap junction formation between Novikoff cells (Meyer et al 1992). The cadherin family of adhesion proteins which are expressed in a tissue specific manner are known to be involved in cell recognition and cell sorting during development (Miyatani et al 1989, Takechei 1991). They may therefore provide a mechanism for segregating cells into separate compartments and restricting junctional communication between cells of different compartments.

Unpublished results by Prowse (1992) showed rat epithelial cells (BRL cells) and rat fibroblasts (BICR cells) sort out into communication compartments in culture. Expression studies revealed differential expression of cell adhesion molecules (but not connexins) and the transfection of E cadherin cDNA into both cell types increased the levels of heterologous communication.

GJIC is often altered during various stages of carcinogenesis. The changes often lead to the restricted communication between normal and transformed cells. Loss of heterologous communication (possibly via altered GJIC specificity) may be a pre-requisite for initiated cells to escape the growth control of normal cells. In several instances changes in GJIC levels are associated with the altered gene expression of cadherin and connexin proteins and in some cases these proteins are post-translationally modified. The situation may be analogous to the formation of communication compartments *in vivo* and the subsequent formation of new cell lineage's within each compartment.

1.4.6. Gap junctional communication and cancer.

Cancer can be described as a disease resulting from altered differentiation and cell growth. Putative roles for GJIC in normal development and growth of organisms have been described in the previous sections. Given the considerable amount of circumstantial evidence for the role of GJIC in growth control it might be supposed that there is a

relationship between cancer and loss of gap junctional communication. There are several lines of evidence to support this:

1). GJIC is often reduced in transformed cells expressing various oncogenes including, ras, src, E5 and raf (Bignami et al 1988a & b, Filson et al 1990, Finbow et al 1991, Kalami et al 1992).
2). The gap junction associated proteins, Cx43 and ductin are the target of the oncogenes src and E5 respectively - although the effects may not necessarily be primary events leading to transformation (Swenson et al 1990, Finbow et al 1991).
3). The aberrant growth of transformed cells is suppressed when cultured with excess normal cells (Stoker 1967), and the phenomenon has been correlated to the presence of heterologous communication (Mehta & Loewenstein 1986).
4). Expression of gap junction associated proteins can normalise the growth of transformed cells (Mehta et al 1991, Jou et al 1993, Chen et al 1995).
5). In several model systems, gap junctional communication is abolished by tumour promoting phorbol esters (Asamoto et al 1991, Roseng et al 1993) and enhanced by tumour suppressing agents such as retinoic acid (Mehta et al 1989, Rogers et al 1990).

Carcinogenesis is thought to be multistep process leading to the sequential loss or impairment of growth control mechanisms. The current view of carcinogenesis begins with a potential growth promoting genetic defect occurring in a single (initiated) cell (Armitage & Doll 1954). If the defective cell divides, then further mutations in subsequent cells may arise which provide the cell with further growth advantages. This stage may be promoted if the cells are exposed to tumour promoting agents such as TPA. Tumours may then progress to more aggressive and malignant forms. The final stage of carcinogenesis is metastasis, which typically involves cells from the primary tumour breaking away, moving to new sites where they may lodge and proliferate - forming secondary tumours. Adhesive interactions have important roles in the process of metastasis and adhesive molecules can act as positive and negative modulators of the metastatic process. E cadherin for example, promotes cell-cell adhesion within the tumour and therefore restricts the ability of individual cells to break away to form secondary tumours (Vleminckx et al 1991, Takeichei 1993). Cells which do break away are at an advantage if they can then attach at a new site rapidly (to avoid elimination in the circulation). Therefore cell surface expression of proteins such as integrins positively promotes metastasis (see Zetter 1993 for review).

In section 1.2 it was shown that the environment of a cell is important to that cell's regulation and phenotype. In 1967 Stoker showed that the growth of polyoma-transformed cells could be inhibited by the presence of excess resting normal cells. Subsequently, this form of inhibition has been correlated with the presence of heterologous communication between the normal and transformed cells (Mehta 1986)

and would appear to show that adjacent cell cytoplasm's can contribute to the environment of a cell and its subsequent regulation.

1.4.6.1. *GJIC during multistage carcinogenesis.*

Many cancer cells show defective gap junctional communication, however, it is not clear at what stage or by what mechanism this occurs. Down-regulation of connexins has been observed in human mammary tumour cells (Lee et al 1992) and post-translational modification of Cx43 has been observed in cell lines transformed with v-src (Swenson et al 1990).

Viral-mediated transformation is often associated with decreased levels of GJIC but different viral oncogenes have varying effects on gap junctional communication. Expression of the oncogenes raf, mos, src, ras, neu and PyMT result in the down-regulation of both homologous and heterologous communication, but v-myc and PyLT do not alter communication levels (Atkinson et al 1981, Atkinson & Sheridan 1984, Atkinson et al 1986, Kalimi et al 1992). The effect of v-src on communication has been correlated with the tyrosine phosphorylation of connexins, (Filson et al 1990). In addition, the bovine papilloma virus-1 oncoprotein, E5, binds to the gap junction protein ductin (Goldstein et al 1992) and the related human papilloma virus oncoprotein, E5, inhibits GJIC. In addition the BPV-4 viral oncoprotein, E8, binds to ductin and communication is downregulated in cells expressing this protein (Facchini et al 1996).

During initiation a genetic mutation may arise which renders the cell 'primed' for transformation. In the mouse skin chemical-carcinogenesis model, in which tumours are induced after skin is treated, first with DMBA followed by several applications of TPA, this initiation is marked by the expression of the H-ras oncogene and a subsequent rise in IP_3 levels (Wakelam 1988). Given the proposed role of GJIC during development it might be suggested that such a cell could be suppressed by the homeostatic pressure of the surrounding normal cells via the loss of excess IP_3.

However, recent findings by Novelli (1996), suggest that some tumours may arise from a polyclonal origin. Novelli found that precancerous cells appeared to be capable of inducing precancerous states in neighbouring cells. These results concerned the apparent polyclonal origin of adenomas and subsequent tumours in the human colon. However, at present there is no evidence to suggest that such a mechanism is involved in the origin other tumours. The molecular and biochemical signals that were involved in the induction have not been characterised, but could be mediated by the transfer of aberrant growth signals via gap junctions.

In culture, normal cells generally cease growing once they have reached high cell density. It has been shown that this inhibitory growth control can suppress the aberrant growth of certain transformed cells, providing there is heterologous communication between the two cell types (Stoker 1967, Mehta et al 1986). The ability of transformed cells to stimulate the growth of resting normal cells is therefore potentially important, i.e.

by altering the local environment to one which promotes growth, the inhibitory effect of the normal cells may be reduced or cancelled out.

Returning to the mouse skin model, the ability of the initiated cell to clonally expand requires several applications of TPA. TPA transiently inhibits junctional communication *in vitro* (Mehta et al 1986 - see section 1.3.4.3 on TPA action) and can release transformed cells from the growth suppression of normal cells (Dotto et al 1988). Thus, the effect on mouse skin is consistent with the idea that the initiated cell must escape the growth control imposed by the normal cells via GJIC (Mensil & Yamasaki 1993), until a large enough population has been established in which cells are less influenced by surrounding normal cells and can grow, acquiring further relevant genetic changes.

Investigations have been carried out to determine whether the changes in GJIC associated with progression correlate to altered levels of gap junction associated proteins. The levels of Cx26, and Cx43 were examined by immunofluoresence in normal mouse skin, papillomas, squamous cell carcinomas (SCC) and associated metastases (Kamibayashi et al 1995). In papillomas Cx26 and Cx43 co-localised in the same gap junction plaques, whereas the two connexins were differentially expressed in normal and surrounding non-tumourous epidermis. In SCC's levels of Cx26 and Cx43 decreased in comparison to normal tissue and papillomas. When SCC's metastasised into lymph nodes, Cx26 was expressed but few cells appeared to express Cx43. In metastatic tumours Cx43 was found to be very low. Although the morphometric connexin expression data do not necessarily represent functional states several studies have shown that the number or size of immunoreactive spots of connexins is closely related to levels of GJIC (Krutovskikh et al 1991, Asamoto et al 1991). Reports by Klann et al (1989) also show that levels of GJIC are reduced in a step-like manner as cells progress towards an increased aberrant phenotype.

The importance of connexin expression and GJIC during tumourigenesis is highlighted further by the studies of Mehta et al (1991), Jou et al (1993), Rose et al (1993) and Chen et al (1995). They have shown that the exogenous expression of connexins in a many different transformed cell lines restores or increases heterologous communication. The growth of these cells is often normalised in culture as indicated by lower saturation densities, longer population doubling times and rounder, flatter cell shape. The molecular mechanisms responsible for these changes are not known. It has been suggested that the increase in junctional communication allows specific growth inhibitory molecules to pass throughout the coupled population.

Exogenous connexin expression has also been implicated in the suppression of transformed cells by normal cells. Here it is thought that the protein is responsible for increasing heterologous communication between the two cell types, and it is the normal cells which then impose growth control on the transformed cells population. The cells also show loss or reduction in their tumourigenic potential *in vivo*.

Further complexity in our understanding of the process of carcinogenesis is added by the ability of adhesion molecules to regulate GJIC (Mege et al 1988, Jungian et al 1991). In multi-stage carcinogenesis, alterations to these adhesion proteins are thought to be involved in the later stages of progression when their loss or modification confers metastatic potential on the tumour. A reduction in junctional communication has been observed during the progression of squamous cell carcinomas to spindle cell carcinomas. This has been correlated to the loss of E-cadherin (Holden et al 1996). Communication in many tumourigenic cell lines is rarely zero and the residual low level may be important for tumour survival and metastasis. It has been shown by Sabban et al (1991) that dye transfer occurs between metastatic tumour cells and vascular endothelium.

1.4.6.2. *Homologous and heterologous communication during carcinogenesis.*

Most work on GJIC and its role in carcinogenesis has concentrated on the changes in homologous communication in transformed cells. Most transformed cells show some residual levels of homologous communication (Kalimi 1992) and this fact is currently being exploited for cancer pro-drug therapies (section 1.3.3). The retention of homologous communication may be of advantage to the transformed cells in later stages of progression. For example, in the establishment of a homeostatic pressure which may contribute to maintaining the aberrant phenotype. However, if normal cells are able to control the growth of transformed cells via junctional communication, loss of heterologous communication would be of selective growth advantage to initiated cells.

Several studies have indeed shown that cells transformed by various agents (including chemicals and oncogene expression) lose the ability to communicate with normal cells (Yamasaki & Enomoto 1985, Mehta et al 1991, Kalimi et al 1992). In subsequent studies attempts have been made to restore communication in these cell lines by transfection with Cx32 or Cx43 (Eghbali et al 1990, Mehta et al 1991, Zhu et al 1991, Rose et al 1993, Chen et al 1995). Restoration of heterologous communication resulted in the normalisation of the transformed cells. This was manifest by reduced proliferative capacity in culture and a non-tumourigenic phenotype *in vivo*. The molecular mechanisms for this phenomenon have yet to be characterised but possible mechanisms are discussed in section 1.6.

Many studies regarding cell-cell interactions, particularly GJIC, and their role in growth control have been carried out in culture. The possibility exists that such events are merely tissue culture artefacts. However, there are examples where dramatic alterations in cell phenotype occur *in vivo,* in response to altered environments. One such example is the phenotypic conversion of embryonal carcinoma cells (EC cells) from a malignant form to one in which the cells participate in normal growth and development (in blastocyts). The phenomenon may serve as a complex *in vivo* analogy to that seen in culture when the growth of transformed cells is suppressed by the presence of normal cells. A brief review of this striking phenomenon is given next.

1.5. Embryomal Carcinoma Cells: an *in vivo* example of extra-cellular, environmental growth control.

1.5.1. General overview.

Teratocarcinomas have proved to be a useful model for studying the effect of cellular environment in the process of cell commitment and differentiation. Teratocarcinomas represent tumours of pluripotential cells and can arise spontaneously in murine testis or ovaries; such tumours are rare in humans - Pierce & Dixon 1959. Teratocarcinomas (or embryonal carcinomas) are composed of disorganised mixtures of well-differentiated tissue, typically epithelia, notochord, cartilage (Pierce et al 1967) and undifferentiated embryonal carcinoma cells which confer malignancy upon the tumour. The term, teratoma is used for teratocarcinomas whose EC-cells have all differentiated and are no longer malignant. Transplantable teratocarcinomas can be experimentally produced by grafting early embryos, germinal ridges or germ layers into adult mouse testis. Such teratocarcinomas have a normal diploid set of chromosomes and are indistinguishable from spontaneous ovarian and testicular teratocarcinomas.

The nature and location of teratocarcinomas led investigators to believe that they arose from cells derived from pluripotent cells in the germ layers of early embryos. Several lines of evidence exist to support such a hypothesis. Germinal ridges or early germ layers from mice homozygous for the *steel* gene lack primordial germ cells and do not form teratocarcinomas when grafted (Sherman 1975). Furthermore the ability of germ layers, when grafted, to produce teratocarcinomas is lost by the 8th day of gestation - a time at which cell commitment is restricted.

Definitive evidence that EC-cells are pluripotent came from a series of experiments through the mid 1960's and early 1970's. Embryoid bodies are teratocarcinomas that form in the peritoneal cavity and consist of a thin sheet of endoderm which surrounds a core of EC-cells (Stevens 1970). In 1964 Kleinsmith and Pierce took single cells from such tumours and grafted them into mice. The teratocarcinomas which subsequently formed contained EC-cells as well as various well differentiated tissues, (up to 14 types). Although clearly pluripotent it was not clear whether such cells were totipotent as the tumours lacked several tissue types. In 1975 it was shown that EC-cells could participate in normal development. Mintz & Illmenses (1975) took a single EC-cell and injected it into a mouse blastocyst whereupon a chimeric embryo developed. These cells contributed to various somatic tissues including liver, thymus, kidneys and germ cells.

These experiments clearly highlight the role of the environment in effecting the programming of the genome. The ability of EC-cells to form normal adult tissue and germ cells (Stewart & Mintz 1981) demonstrates that the conversion to neoplasia under certain conditions does not involve permanent structural changes to the genome but rather a change in gene expression in response to altered environments. Evidence from

other sources also highlight the importance of such environmental interactions in determining cell phenotype. For example, epithelial cell carcinomas can revert to a non-tumourigenic phenotype after interaction with normal skin (Cooper & Pinkus 1977). In this instance a genetic change is being suppressed.

The interactions which determine cell phenotypes in teratocarcinomas are still not understood, although progress has been made in understanding, at a molecular level, the changes which result in a switch from a neoplastic to a normal cell phenotype. Retinoic acid (RA) has been well studied as a mediator of inductive signals during EC-cell differentiation. The inductive ability of RA has been shown using an EC-cell line, F9 cells, which have lost the ability to differentiate *in vitro* or *in vivo* (Strickland & Mahdave 1973). The RA-associated molecular changes which drive the differentiation of F9 cells to a new phenotype are now being dissected. A variety of transcription factors have been identified which show altered levels of abundance and activity in response to RA treatment. These factors (which include c-jun & AP-1) modulate the activity of genes responsible for cell proliferation and ECM production (Sleigh 1992).

The role of GJIC in differentiation of EC-cells has not been fully explored. Gap junctions certainly provide an apparatus for establishing and maintaining microenvironments which may be important in embryogenesis and the maintenance of the differentiated state. Using F9 cells it was first demonstrated that gap junction associated genes can be modulated during the differentiation of a single cell type in culture (Nishi et al 1991). Upon exposure to RA, the F9 cells rapidly differentiated into visceral endoderm (VE) or parietal endoderm (PE). All cells expressed Cx43. Cx26 was also expressed in all cells although differentially i.e. PE cells had very low expression and VE cells had very high expression levels. Cx32 was only expressed in VE cells. The modulation of gap junction associated genes during differentiation in this system is in agreement with the analysis of fully differentiated tissue *in vivo*. Nicholson et al (1987) found that foetal liver predominantly expressed Cx32 and Cx26, and VE cells in teratocarcinomas are thought to be the equivalent of foetal liver precursor cells (see Nicholson 1987 and references there-in). Causal relationships between gap junctional communication deficiency and loss of EC-cell differentiative ability has also been suggested by Sheardown & Hooper (1992).

1.5.2. Summary.

The occurrence of teratocarcinomas would appear to be due to the ability of stem cells to retain their proliferative capacity. This ability appears to be the result of general tissue disorganisation during development. In a normal environment the aberrant growth phenotype of the stem cells can be completely reversed and it has been shown that this is not the result of selecting non-malignant cells (Mintz & Illmensee 1975). The experiments which show that previously malignant EC-cells can undergo normal differentiation and contribute to the normal growth of a mouse demonstrates that their

initial state of malignancy was due to a change in gene expression rather than a permanent structural change to their genomes.

Although EC-cells and teratocarcinomas have proved a useful model for studying cell differentiation and the importance of the environment in altering gene expression (and hence cell phenotype) the model is a particularly complex one. The fact that the phenomenon occurs *in vivo* makes it difficult to dissect the individual mechanisms which may be involved. However, a similar phenomenon, in terms of the importance of environment in altering cell phenotype, has been observed in culture i.e. the suppression of transformed cell growth by normal cells. The phenomenon is particularly amenable to experimentation.

1.6. Suppression of the transformed phenotype by normal cells.

The observation that the aberrant growth of polyoma virus transformed cells could be inhibited when grown in the presence of excess normal fibroblasts (Stoker 1966 & 1967) provides an *in vitro* system to examine the importance of environment in determining cell phenotypes. The inhibition occurred after the normal cells had reached high density and were themselves, growth inhibited.

A great deal of information regarding the inhibition phenomenon was obtained by Stoker in the original papers (Stoker 1966 & 1967) using a technique which permitted the level of growth inhibition to be measured ([^{3}H]-thymidine incorporation and analysis by autoradiography). It was shown that the growth of the transformed cells was reduced in response to a decrease in the growth of the normal cells when they reached high cell density. The effect was not due to nutrient deprivation or factors secreted into the media. Contact between the two cell types was required and it was shown that there was heterologous metabolic co-operation between the two cell types, via gap junctions. From this data it was hypothesised that the inhibitory effect of the normal cells could arise from the transfer of inhibitory growth signals from normal to transformed cells. The level of normal cell proliferation was higher than background in some of the co-cultures, however, this was not quantified.

In 1986 the growth inhibition of various chemically and virally transformed cells was shown to correlate to the level of heterologous gap junctional communication with surrounding normal cells (Mehta et al 1986). In this work the proliferation assay, used by Stoker to quantify inhibition, was neglected in favour of the quicker, though less informative, focus formation assay. Mehta et al reached the same conclusion as Stoker, that is, inhibitory molecules *may* pass from the normal to the transformed cells. However, such molecules have yet to be identified. Subsequent published results have confirmed the inhibitory effect normal cells can have upon the growth phenotype of transformed cells (Bignami et al 1988, Dotto et al 1988, Martin et al 1991, Crescenzi & Tato 1994). La Rocca et al (1989) found that in addition to suppressing the aberrant

growth phenotype of myc-transformed quail muscle cells, contact with excess surrounding normal fibroblasts were capable of reverting the cells back to their myogenic differentiation program.

These studies, based on a variety of cell systems, used the focus formation assay as a means of detecting whether the normal cells are capable of suppressing the transformed cells. In a typical assay a small number of transformed cells are cultured together with an excess of normal cells or seeded on top of a confluent layer of normal cells. After 1-2 weeks in co-culture the number of foci are counted and/or their size measured and inhibition calculated. However, it provides little other information. Individual cell-cell interactions cannot be observed and suppressed cells cannot be studied (because they cannot be identified). Any subtle changes in growth, such as the stimulation of normal cells, which Stoker (1967) suggests may occur, cannot be detected because no proliferative data, other than indirect measurements gained from focus number or size, is obtained. Furthermore, focus assays provide little scope for *in vivo* studies. Tumourigenicity assays can be carried out, but again, only cells which have escaped suppression and formed tumours can be examined. Such assays raise further questions; for example, why is it necessary to inject such a large mass of cells ($>10^3$ cells in even highly tumourigenic cell lines) before tumours are detected? And furthermore, what happens to the cells which don't go onto to form tumours - are they suppressed? If they are suppressed until they eventually die, this may be an important defence against cancer.

Attempts have been made to study the role of GJIC in the inhibition phenomenon by experimentally varying the level of communication. However, the agents used in such treatments (e.g. forskolin and retinoids - Mehta et al 1986, 1989, Mehta & Loewenstein 1991) are insufficiently specific modulators of junctional communication and specific conclusions are difficult to draw.

1.6.1. Mechanisms of suppression.

The ability of a normal cell to crawl and spread out over a substratum to occupy vacant space strongly affects its ability to divide. The frequency with which a cell divides increases as the cells become more spread out, (O'Neill et al 1986; possibly because a cell with a larger surface area can take in more nutrients and growth factors). Some transformed cells will divide even when the adhesion site is small and leaves no room to spread out. Normal anchorage-dependent cells in culture are characterised by their density-dependent regulation of growth (Wieser et al 1990). The inhibition of transformed cells by normal cells occurs once the normal cells have reached high density and have themselves stopped growing, it is possible that the same growth control mechanisms are responsible for both these phenomena. However, the specific molecular mechanisms are not known.

The mechanism responsible for the growth suppression does not appear to involve a secreted factor, based on the failure to transfer inhibition using conditioned media and the requirement for direct cell-cell contact. However, a secreted factor may act in a paracrine fashion i.e. requires high local concentrations for effectiveness due to rapid internalisation by surrounding cells, binding to the surface or ECM receptors of other cells or rapid degradation. The high concentration required may be difficult to achieve using standard conditioned media experiments.

Inhibition models have been proposed which are centred on either the production of specific growth inhibitory molecules by normal cells or the dilution of stimulatory factors produced by the transformed cells, both processes mediated by gap junctional transfer (Stoker et al 1967, Mehta et al 1989, Mehta et al 1986). As yet, however, no specific inhibitory molecules, which could pass through gap junctions, have been found. GJIC may induce suppression by a more general mechanism such as homeostasis. A general equilibration of small molecules and ions between the two cell types may result in a homeostatic pressure which suppresses differences between cells.

The studies in which connexins were transfected into communication deficient transformed cells to restore GJIC and a normal phenotype would appear to provide direct evidence that the inhibition is mediated by GJIC. However, it has not been unequivocally shown that connexin expression only effects GJIC. Furthermore, many investigators believe that connexins exclusively form the channel component of the connexon but this has not been shown and they may serve other functions which in turn regulate GJIC. Cadherins for example are known to effect GJIC but it is not suggested they form part of the channel structure.

Overexpression of Cx43 in transformed cells, which can cause the cells to revert to a normal phenotype, has been shown to alter the expression levels of specific genes involved in cell cycle regulation - namely cyclins and cyclin dependent kinases (Chen et al 1995). The authors were unable to distinguish between the expression of Cx43 versus the restoration of GJIC as being responsible for the effects observed on cell phenotype. The relationship between Cx43 expression and cell cycle gene expression may arise from a direct exchange of growth regulatory factors through gap junctions or via the interaction of Cx43 on adjacent cells which may directly affect cell signalling or cell adhesion pathways responsible altering specific genes.

Density-dependent inhibition of cell growth has been correlated to the expression of a glycoprotein termed contactinhibin which binds via cell-cell contact to its appropriate contactinhibin receptor (CiR) on apposing cell membranes (Weiser et al 1990, Gradl et al 1995). However, it is not known at present whether this form of inhibition is responsible for that seen by Stoker and others.

1.7. Summary and aims.

There is abundant circumstantial evidence to suggest that GJIC is involved in the control of normal cell growth and development. However, the nature of the signal molecules and the control pathways affected remains unclear. The importance of environment in altering gene activity has been stressed throughout and clearly demonstrated by the altered fates of embryonal carcinoma cells in response to different environments. Gap junctional communication is likely to be just one of a series of important cell-cell interactions involved in defining and maintaining various differentiated phenotypes, and may provide a general means of co-ordinating cellular activities.

The observations by Stoker (1967) have demonstrated further the idea of environment controlling cell growth and phenotype. In this instance subsequent studies have implicated GJIC as a likely mediator of the growth control, transferred from the normal cells to the transformed cells *in vitro*. However, because most studies have been concerned with the role of GJIC in carcinogenesis detailed studies of the inhibition phenomenon *in vitro* or *in vivo* have not been carried out.

Given that such a phenomenon may play an important role as a primary means of defence against cancer this thesis aims to provide a detailed examination of the phenomenon *in vitro* and establish an assay which can be subsequently used *in vivo* to assess the role of the inhibition phenomenon during the process of tumourigenesis. The potential of such assays was recognised by Stoker (1967) who used lineage marking techniques and a proliferation assay based on thymidine incorporation to examine the suppression of the transformed phenotype by normal cells. Despite all of the work carried out there has been little advance in the detailed description or understanding of the phenomenon since these earlier investigations.

CHAPTER TWO

MATERIALS & METHODS.

2.1. Materials.

2.1.1. Chemicals.

Chemicals used were of "AnalR" grade and obtained from BDH Chemicals Ltd., Poole, Dorset, England or Sigma Chemicals Co., Ltd., Poole, Dorset, England, except those from the suppliers listed below.

SUPPLIER	CHEMICAL
Amersham International Plc., Aylesbury, Bucks, UK.	$\alpha[^{32}P]$-dCTP (3000Ci/mmol) [Methyl-^{3}H]-thymidine (25 Ci/mmol) [5-^{3}H]-uridine (29 Ci/mmol) Hybond-ECL, Hybond-N^{+}
Boots Plc, Argyll St, Glasgow.	Clear nail varnish
BRL (UK), Gibco Ltd., Paisley, Scotland.	All restriction enzymes and buffer
Biogenesis Ltd., Bournemouth, England.	RNAzol B.
Boehringer Mannheim UK Ltd., Lewes, East Sussex, England.	DNA molecular weight markers IV and VI Proteinase K.
J. Burrough (FAD) Ltd., Witham, Essex, England.	Ethanol.
Central Services, Beatson Institute.	Sterile distilled water and PBS L-broth, CT buffer (NaCl 6g, Trisodium Citrate 2.96g, Tricine 1.76g, Phenol red 5mg, Water 700ml, pH7.8), PBS, Glycerol.
Difco Labs., Detroit, Michigan, USA.	Bacto-agar. Bacto-tryptone.
Gateway Plc, Glasgow Scotland.	Marvel Dried non-fat milk powder.

Oxoid Ltd., Basingstoke, England.	PBS tablets.
Pharmacia Biotech Ltd., Milton Keynes, England.	Micro spin columns s-400 HR
Rathburn Chemicals Ltd., Walkerburn, Scotland.	Phenol (water saturated)
Sigma Chemical Co., Ltd., Dorset, England.	Pre-stained protein molecular weight markers
Vector Labs Inc. Burlington, USA	Vector shield mounting media.

2.1.2. Kits.

SUPPLIER	KIT
Amersham International Plc. Bucks, England.	ECL Western Blotting Analysis System. Cell Proliferation Kit
Bio 101 Inc. Luton, England	Geneclean Kit
Boehringer Mannheim UK Ltd., Lewes, East Sussex, England.	Apoptosis, cell death detection kit
Molecular Probes, Cambridge Bioscience, Swanns Rd, Cambridge, England	Imagene *lac*Z Expression Detection Kit. FluoReporter *lac*Z Quantitation Kit. Fluorescent microspheres
Pharmacia Ltd, Milton Keynes, England .	Oligo-labelling Kit.

2.1.3. Water.

Distilled water for solutions was obtained from a Millipore MilliRO 15 system, and for nucleic acid procedures it was further purified on a Millipore MilliQ system.

2.1.4. Equipment and Plasticware.

Main pieces of equipment are referred to in the appropriate sections. The suppliers of the most commonly used items are listed below:

SUPPLIER	EQUIPMENT
Beckton Dickinson Labware, Plymouth, Devon, England.	Tissue culture dishes (35, 60, 90mm)
Bibby-sterelin Ltd, Stoney, Staffs., England.	Bacteriological dishes (90 mm)
Chance Propper Ltd., Warley, England.	16-22mm glass coverslips
Clarke Electromedical Instruments, Reading, England.	Thin wall glass capillaries.
Costar, Cambridge, Masachusetts, USA.	96 well plates, Disposable cell scrapers.
Eastman Kodak Co., Rochester, New York, USA.	X-ray film (XAR-5)
Fuji Photo Ltd, Japan.	X-ray film (RX)
Gibco Europe, Life Technologies, Paisley, Scotland.	Nunc 1ml cryotubes, Chamber slides (8, 4, 2, 1 well), Tissue culture flasks (25, 80 and 175 $cm^{3)}$
Griener Labortechnik Ltd., Dursley, England.	Eppendorf tubes, Pipette tips (200 and 500ul)
Whatman International Ltd., Maidstone, England.	3mm chromatography paper.

2.1.5. Antiserum.

SUPPLIER	ANTIBODY
Takara Shozo Co., Ltd., Seta, Otsu, Japan.	Mouse monoclonal anti-E cadherin (ECCD-1 and ECCD-2) Mouse monoclonal anti-P cadherin (PCD-1)
Sigma Chemical Co., Ltd., Dorset, England.	Mouse monoclonal anti-Ncadherin (GC4) Mouse monoclonal anti-N CAM (OB11). Goat anti-mouse IgG FITC conjugate. Goat anti-rabbit IgG FITC conjugate. Goat anti-rat IgG FITC conjugate. Rat monoclonal Anti-Uvomorolin (DECMA1).
Zymega Reasearch, San Francisco, CA 94080 USA.	Mouse monoclonal anti-connexin 43 Mouse monoclonal anti-connexin 32 Mouse monoclonal anti-connexin 26
Amersham International PLC., Bucks., England.	Sheep anti-mouse hp-linked whole antiybody. Sheep anti-rabbit hp-linked whole antiybody. Sheep anti-rat hp-linked whole antiybody.

2.1.6. Plasmids and bacterial host.

Compotent E.coli DH5α were obtained from B.R.L., Gibco Ltd.

pSVL - β-gal expression vector. A gift from S. Frame, Beatson Institute.

pNeo - neo resistance expression vector. B.R.L., Gibco Ltd.

2.1.7. Cell Culture Materials.

SUPPLIER	MATERIAL
Life Technologies Ltd (Gibco Europe), Paisley, Scotland.	2.5% (w/v) Trypsin Penicillin / streptomycin / gentamycin / Amphotericin 200mM Glutamine 7.5% Sodium Bicarbonate 100mM Sodium pyruvate Hepes Buffer DMEM x10
Northumbria Biologicals.	Foetal calf serum
Sigma Chemical Co., St Louis, USA.	Trypan blue

2.1.8. Cell lines.

CELL LINE	DESCRIPTION
Rat2	Normal rat fibroblast, TK defficient*
R24E	Rat2 - E cadherin transfectant*
10T1/2	Normal mouse fibroblast
S180	Mouse sarcoma derived fibroblast*
S180E217	S180 E cadherin transfectant*
S180NII	S180 N cadherin transfectant*
S180N2A1	S180 N cadherin transfectant*
BICR	Rat mammary carcinoma fibroblast
SVT2	3T3 - SV40 transformed cell line

* Cell lines were a gift from Prof. F. Tato, Rome, Italy.

2.2. Tissue Culture Techniques.

2.2.1. Cell maintenance.

Cell lines used throughout this thesis are listed in the materials section 2.1.8. All cell lines were maintained in DMEM, which was supplemented with 10% (v/v) foetal calf serum, (unless stated otherwise) and 2mM L-glutamine. This media is referred to as DMEM10%. The following cell lines were maintained in DMEM10% supplemented with 500μg/ml hygromycin: S180, S180E217, S180NII, S180N2A1, R24E. Cell lines which were transfected with the β-gal expression vector were maintained in media containing 500ug/ml G418.

Cells were routinely sub-cultured every 4 days unless stated otherwise in the text or figure legends. The cells were cultured in 90mm tissue culture plates in a 7% CO_2 atmosphere at 37°C. Maintenance of the cells was carried out in a laminar-flow-hood. All of the cell lines were routinely examined for mycoplasma infection and were consistently negative.

2.2.2. Measurement of growth parameters.

2.2.2.1. *Determination of cell viability*

Cell viability was determined by mixing 25μl of cell suspension with 75 μl of 0.05% (w/v) trypan blue in PBS. The membranes of dead cells lose their integrity allowing trypan blue to enter the cell. The numbers of live and dead cells were counted using a Neubauer haemocytometer. The total number of cells in 4 x 16 square grids was multiplied by 10^4 to estimate the number of cells per ml of culture:

$$\% \text{ viability} = (\text{number of live cells} / \text{total number of cells}) \times 100$$

2.2.2.2. *Population doubling time.*

Cells were seeded at 5 x 10^5 cells per 90mm dish. The number of live cells (using trypan blue to determine cell viability) were counted every 12 hours using a haemocytometer. Growth curves were plotted, from which population doubling times were determined.

2.2.2.3. *Saturation density.*

Cells were seeded at 5 x 10^5 and counted every 12 hours. Saturation density was considered to have occurred once the size of the cell population no longer increased. The labelling index (the proportion of cells incorporating [^{3}H]-thymidine) of the cultures was taken every 24 hours (see section for 2.5 procedure).

2.2.2.4. Growth in low serum.

Cells were plated at 5 x 10^5 per 90mm dish in normal media and left overnight to attach. The media was removed and the cells washed twice with PBS. Fresh, low serum media, (0.5%FCS) was added and the cell number counted every 12-18hrs.

2.2.3. Transfection of cell lines with a β-gal expression vector.

Transfections were performed using the GIBCO Lipofectamine™ transfection kit following the procedure below. Cells were co-transfected with the plasmids pSVl (β-gal expression vector) and pNeo (plasmid containing a G418 resistance gene). Cells expressing the pNeo vector were selected in media containing G418 at the appropriate concentration (500μg/ml for the majority of cell lines with the exception of Rat2 cells: 900μg/ml).

- Exponentially growing cells were seeded at $3x10^5$ cells per 35mm dish in DMEM10% and left for 24hours at 37°C in an atmosphere of 5% CO_2.
- 10μl of lipofectamine was mixed with 100μl of serum free media in a 1ml eppendorf. In a separate eppendorf 100μl of serum free media was mixed with 1μg of pNeo and 10μg of pSVl. The contents of the two eppendorfs were mixed and left for 45 minutes or until a precipitate formed. The precipitate was added to the cells and left overnight. Two mock control mock transfections were set up, one in which only pNeo was transfected and one in which plasmids were replaced by an equal volume of PBS.
- The following day the media was removed and the cells washed with PBS. Fresh media was added and the cells left for a further 24 hours. At this point the cells were split at 1:10 into 10, 90mm petri dishes and 10mls of selection media added. This selection regime was applied for 2-3 weeks, and the media replace every 3 days. Resistant colonies appearing over this time were ring cloned, (see below).

2.2.3.1. Ring cloning of resistant colonies.

- The position of G418 resistant colonies were marked on the bottom of the culture dish and the media removed. Cells were washed twice with PBS. Sterile rings were carefully applied to the marked colonies, (rings were prepared by cutting the end of yellow pipette tips with a scalpel, autoclaving and greasing the base with a small amount of sterile Vaseline prior to use).
- 50μl of trypsin was added to each ring and left for 1-3 mins. The cells were resuspended in the trypsin and added to 1ml of normal medium in a well of a 24 well plate.

• Successful ring cloned colonies were maintained in media containing G418 at appropriate concentrations and expanded by subcloning into 90mm dishes.

2.2.4. Preparation of conditioned media.

Conditioned media was prepared by growing cultures to confluence in DMEM10% medium. Conditioned media was removed after 10 days and centrifuged at 3000xg for 10 min to remove debris and used directly or stored at -20°C (for a max. of 7 days).

2.2.5. Frozen cell stocks.

Frozen cell stocks of each cell type were maintained in liquid nitrogen. Cell lines were trypsinised and resuspended at 10^6 cells per 1ml of appropriate media supplemented with 10%DMSO. The cell suspension was added to 1ml Nunc cryotubes, frozen at -70°C overnight and transferred to liquid nitrogen vats for long term storage.

2.3. Gap junctional communication assays.

2.3.1. Dye transfer.

Levels of homologous communication were measured by micro-injecting the cells with the tracer dye Lucifer Yellow CH. Cells were grown in 60mm tissue culture dishes until 80-100% confluent. Immediately prior to the dye injection the media was removed and replaced with fresh media buffered with 25mM Hepes (in place of Sodium Bicarbonate). The cells were then transferred to a 37°C microscope stage and the lid of the dish removed. An individual cell was selected and iontophoroetically injected with dye using micro electrodes made from "kwik-fill" thin-wall glass capillaries which were filled with 10µl of 4% Lucifer Yellow CH as described by Pitts and Kam (1985). The cells were injected with dye for 2 mins using a current of 10nA in 0.5 second pulses at 1Hz and the process monitored using a Leitz Diavert inverted microscope with UV (epi-illumination) or visible (phase contrast) light sources. The extent of dye spread to neighbouring cells was recorded and photographed immediately.

2.3.2. Nucleotide transfer

Nucleotide transfer was used to examine the levels of heterologous and homologous communication amongst the cells lines selected. The technique of nucleotide transfer is based on the transfer of [^{3}H]-nucleotides (derived from [^{3}H]-uridine) through gap junctions. The labelled nucleotides are incorporated into the

RNA of coupled cells and the extent of transfer can be analysed using autoradiography.

• 2.5 x 10^5 exponentially growing cells representing the recipients were seeded onto a single well chamber slide. 1.5 x 10^5 cells, cells representing the nucleotide donor cells (the same cell line as the recipients for homologous communication and a different cell line if studying heterologous communication) were seeded onto a separate single well chamber slide.
• When the recipients were 70-80% confluent, the donor cells were pulsed with 0.46MBq of [^{3}H]-uridine per ml of media for 4 hours. Cells were then washed 4 times with unlabelled medium and trypsinised. After 2-3 mins the trypsin was removed and the cells resuspended in 2mls of DMEM10%. Approximately 10µl of this suspension was added to the recipient cells and incubated at 37°C for 4 hours.
• The co-cultures were then washed 3 times with PBS and fixed in formal saline (0.9% NaCl, 10% Formaldehyde) for 30 mins.
• Cultures were briefly washed with PBS followed by a 5 minute incubation in cold 5% trichloracetic acid (TCA). Cultures were then submerged into gently running cold water for 30-60 mins, washed once with cold ethanol and allowed to dry prior to autoradiography (see section 2.5.2 for autoradiography procedure).

2.4. Focus formation assay.

In a basic focus formation assay normal and transformed cells were plated at 10^6 and 500 cells per 90mm dish respectively. Controls were represented by separate cultures of each cell type seeded at the same densities as above. All experiments were set up in at least duplicate. Once experiments were set up, cultures were maintained in a humidified incubator at 37°C. After 12 days cultures were fixed in 1% formaldehyde and 0.1 % gluteraldehyde in PBS they were then stained with x-gal for 8 hours (section 2.5.1) and counterstained with Giemsa (1:10) for 10 mins. The number of colonies or foci per plate were counted and their average size (area) determined. In initial experiments a minimum threshold size of 0.4mm (0.125mm^2) was used as a cut-off point below which colonies were neither counted or measured. Deviations from this basic format are described in detail where appropriate in the results text and figure legends.

In some focus assays the average focus cell density was measured. Cell density is given as the number of cells / UA. A UA of 0.175mm^2 was chosen as this was the size of the eye piece grid and large enough to obtain sufficient data with statistically confidence. Average focus cell density was obtained from examining 5 foci and 5

respective control colonies per plate. For each focus cell density was derived from 10 UA's (or the maximum possible when focus size was small).

2.5. Proliferation assay.

Focus assays were set up in the same way as the focus formation assay unless stated otherwise. However, 18 hours prior to fixing, the cells were pulsed with [^{3}H]-thymidine (0.185MBq/ml). The media was removed and the cells washed 3 times with PBS. Cultures were fixed in 1% formaldehyde and 0.1% gluteraldehyde in PBS and stained in x-gal for 8hrs (section 2.5.1). The cultures were then submerged in a 5% solution of TCA for 5 mins, washed in running water for 45 mins and rinsed with cold ethanol. The cells were then exposed to photographic emulsion following the procedure in section 2.5.2.

2.5.1. Staining procedure for cells expressing the β-gal lineage marker

Prior to staining the following solutions were prepared:

Fixative: 1% formaldehyde and 0.1% gluteraldehyde in PBS.

Stocks for staining solution:

a). 40mg/ml of 5-Bromo4-Chloro3-Indolyl β-D-Galactopyranoside (x-gal) in DMSO. Prepared before use.

b). 0.5M K_3Fe (CN_6), (Potassium Ferricyanide). 16.42g in 100 mls dH_20.

c). 0.5M K_4Fe (CN_6)$3H_20$, (Potassium Ferrocyanide). 21.12g in 100mls dH_20.

d). 1M $MgCl_2$ $6H_20$

Staining Solution:

To make 10mls of stain, sufficient for one 90mm plate the following solutions were added to a universal in the order shown.

9.45mls PBS, 250µl of (a) + 100µl of (b) + 100µl of (c) + 20µl of (d).

Cultures were incubated in the fixative for 30 mins and washed once with PBS. The cultures were then incubated for 8hrs at 37°C in the staining solution detailed above. Those cells that expressed β-gal turned a dark blue, the staining solution was removed, and the cultures washed twice with PBS and the cells either counterstained in a 1:10 solution of Giemsa for 10 mins, washed twice with PBS and mounted in 50% PBS/Glycerol or exposed to photographic emulsion for autoradiographic analysis (section 2.5.2).

2.5.2. Autoradiography of cells exposed to [^{3}H]-uridine or [^{3}H]-thymidine

Cells pulsed with [^{3}H]-thymidine or [^{3}H]-uridine were exposed to photographic emulsion following the procedure detailed below.

- A stock solution of Ilford-K5 photographic emulsion was prepared (in total darkness) by melting 100mls of emulsion at 40°C for 25mins, and diluting with 33mls of warm water, (this solution was stored in complete darkness at 4°C and re-melted prior to use).
- TCA treated cells on dishes or slides were, in total darkness, coated with a thin layer of this stock solution. Approximately 3mls of the molten K5 solution was applied to a 90mm plate, whilst slides were dipped in to a slide mailer containing 15mls of emulsion, the excess poured off and the plates / slides stored in a light proof box with a LARGE sachet of silica gel. After 5-7 days the slides or plates were developed:
- In complete darkness, slides and plates were submerged in D19 Kodak developing solution for 5 mins followed by washing in cold water for 1 minute. Samples were then submerged for a further 5 mins in a solution of 1:10 Ilford Hypam fixer, followed by a final wash in water at which point samples were either stored or counter stained with Giemsa (1:10).

2.6. Apoptosis assay.

An in situ cell death detection kit (supplied by Boehringer Ltd; 2.1.2) was used to determine if cells in culture were undergoing apoptosis. In general, cells entering an apoptotic state undergo a characteristic pattern of structural changes in the nucleus and cytoplasm. A rapid degradation of the plasma membrane is associated with extensive damage to chromatin and DNA cleavage into oligonucleosomal length DNA fragments. The kit utilises immunocytochemical detection of apoptosis at the single cell level based on in-situ labelling of apoptosis induced DNA strand breaks. The procedure below was followed:

- Cultures were prepared in 90mm dishes (see figure legends for details), grown for desired period and fixed in freshly prepared 4% paraformaldehyde in PBS (pH7.4) for 30 mins at room temperature.
- Cells were then permeabilised in a 0.1% Triton X-100 in 0.1% sodium citrate solution for 20 mins. They were then rinsed twice with PBS and 50µl of TUNEL reaction mixture (Terminal deoxynucleotidyl transferase-mediated nick end labelling) was applied for 60 mins. To prevent evaporative loss of the TUNEL solution samples were covered with coverslips and incubated at 37°C in a humidified chamber.
- After rinsing the samples 3 times with PBS they were mounted in 50% glycerol/PBS and viewed under UV light using a Leizt vario orthomat microscope.

• A negative control was included in each experiment - fixed and permeabilised cells were incubated with Label solution (supplied) rather than TUNEL, incubated at 37°C for 60 mins and viewed under UV illumination. As positive controls, cells were treated 100µl of DNase 1 (supplied) for 10 mins prior to rinsing in PBS and application of TUNEL.

2.7. Nucleic acid procedures.

2.7.1. Growth, transformation and storage of competent cells.

The competent *E.coli* strain DH5α, (which is suitable for transformation with plasmid DNA) was purchased from B.R.L. and stored in 20µl aliquot's at -70°C.

• Cells to be used in the transformation were thawed on ice and gently mixed. To each 20µl of competent cells 100ng of DNA (in a final volume of 100µl) was added and incubated on ice for 30 mins. The DH5α cells were then heat shocked at 42°C for 45 seconds followed by incubation for 2 mins on ice, after which 80µl of SOC medium (2% bacto-tryptone, 0.5% bacto-yeast extract, 9mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 20mM glucose) was added.

• To allow the expression of the antibiotic resistance marker, (ampicillin) the cells were transferred to an incubator and shaken for 1hr at 37°C. The cells were then spread onto agar plates (1.5% agar in L-broth) containing 100mg/ml of ampicillin and the plates incubated upside down at 37°C overnight to allow for the formation of colonies.

• Sterile pipette tips were used to pick colonies which were then transferred to a universal container containing 3ml of L-broth and 100mg/ml of ampicillin and incubated for 4-6 hours at 37°C in a shaking incubator. From this solution subsequent mini or maxi plasmid preps could be made.

• Glycerol stocks were prepared from DH5α cells containing the plasmid required by mixing 1ml of DH5α culture with 1ml of 40% glycerol and frozen in Eppendorf tubes at -70°C.

2.7.1.1. *Large scale plasmid preparations (Qiagen).*

• 3ml of the bacterial solution prepared in section 2.7.1. was transferred to a 500ml L-broth culture, containing 50µg/ml ampicllin and left shaking overnight at 37°C.

• The bacteria were pelleted by centrifugation at 5000rpm for 10 mins in a Sorval centrifuge (GS-3 rotor) and the supernatant removed. To obtain a preparation of approximately 1mg plasmid DNA per ml the following procedure was followed:

Solutions and buffers, supplied in the Qiagen kit.

P1- 50mM tris, 10mM EDTA, RNAse 100μg/ml, pH 8.0

P2- 0.2M NaOH, 1% SDS

P3- 2.55M KAc, pH8.4

QBT- 750mM NaCl, 50mM MOPS, 15% ethanol, 0.15% Triton X-100

QC- 1M NaCl, 50mM MOPS, 15% ethanol, pH7.0

QF- 1.25M NaCl, 50mM MOPS, 15% ethanol, pH8.2

Procedure:

• The bacterial pellet was resuspended in 10ml of buffer P1, to which 10ml of buffer P2 was added and incubated at room temperature for 5 mins.

• 10ml of P3 buffer was mixed with the above solution and incubated on ice for 20 mins. The precipitated material was pelleted by centrifugation at 4°C for 30 mins at 10000rpm in a Sorval centrifuge (HB-4 rotor). A Qiagen-tip 500 column was equilibrated with 10ml of buffer QBT and the supernatant promptly applied to it.

• The column was washed twice with QC buffer and the plasmid DNA eluted by passing 15ml of QF buffer through the column.

• The DNA was precipitated with 0.7 volumes of isopropanol and pelleted by centrifugation at 10000rpm at 4°C for 30 mins in a Sorval centrifuge (HB-4 rotor). 70% ethanol was used to wash the DNA pellet which was then allowed to air dry before being redissolved in 500μl of water and stored at -20°C.

2.7.2. Determining nucleic acid concentration.

The concentration of nucleic acid in a solution was determined spectrophotometrically, by first calibrating the spectrophotometer (Beckman) using a water blank and then diluting the samples in water and reading the absorbency at wavelengths 260nm and 280nm in a quartz cuvette with a pathway of 1cm. For plasmid and genomic DNA samples an A260 of 1 was taken to correspond to 50mg/ml and for RNA an A260 of 1 was taken to correspond to 40mg/ml.

2.7.3. Agarose gel electrophoresis.

• Gels were prepared by dissolving 1g of agarose in 100ml of 1x TBE buffer (5x TBE is 0.45M Tris, 0.45 M boric acid, 12.5mM EDTA, pH8.3) and boiling until the agarose dissolved. The gel mix was allowed to cool to approximately 50°C before ethidium bromide was added, to a final concentration of 1mg/ml.

• The gel was cast and allowed to set at room temperature before the comb was removed and the gel placed in the gel tank.

• The DNA samples and DNA standards (Boehringer Mannheim) for electrophoresis were mixed with 1/6 vol of DNA gel loading buffer, loaded and resolved by electrophoresis. Electrophoresis was carried out in 1x TBE buffer and run for 30-60 mins at 100v.

• On completion of electrophoresis the size of the DNA fragments were assessed by illuminating the gel on a 312nm transilluminator and compared to the positions of the DNA standards. A digital and photographic record of the UV illuminated gel was taken on a Appligene imager.

2.7.4. Extraction of RNA from mammalian cells.

RNA was extracted from the cells of interest using the Biogenesis RNA isolation kit RNAzol-B. Samples obtained were used directly for Northern analysis or purified further to obtain mRNA (section 2.7.4.1).

• The cells to be assayed were grown to approximately 80% confluence on 90mm dishes. They were then scraped and homogenised (using a cell scraper) in 0.8ml of RNAzol B.

• The homogenates were transferred to a sterile 1ml eppendorf tube to which 80µl of chloroform was added. The tubes were stored for 5 mins at 4°C and then centrifuged for 15 mins at 12,000g.

• The aqueous phase was collected and RNA precipitated with 0.4ml of isopropanol for 45mins. The RNA was pelleted by a 15 minute spin at 12,000g and washed once with 70% ethanol. Once the ethanol was removed the RNA was resuspended in 30-100µl of water, quantified and stored at -20°C.

2.7.4.1. mRNA purification.

RNA extracted from mammalian cells were further purified to obtain mRNA using the DYNAL™ purification kit. Solutions and procedures are listed below:

Solutions and buffers.

2X Binding buffer:20mM Tris-HCL pH7.5, 1M LiCi, 2mM EDTA in a volume of 2ml.

1X Washing buffer: 10mM Tris-HCL pH7.5, 0.15M LiCi, 1M EDTA in a volume of 2ml.

1X Elution Buffer: 2mM EDTA pH7.5 in a volume of 2ml.

Procedure:

• 100µg of total RNA was diluted into 100µl of water and heated to 65°C to disrupt the secondary structures. 0.2ml of dynobead suspension was aliquoted to a 1ml eppendorf and placed in the Magnetic Particle Holder (MPH), supplied. After 30 secs the supernatant was removed and discarded leaving behind the beads.

• The eppendorf was removed from the MPH and the beads washed with 100µl of 2x binding buffer. A further 100µl of binding buffer was added and mixed gently with the RNA / bead solution. This suspension was left to stand for 3 mins at room temperature.

• The eppendorf was returned to the MPH and the supernatant removed. The remaining beads were washed twice with 200µl of washing buffer and RNA eluted from the beads by the addition of 20µl 1X Elution buffer. This solution was heated to 65°C and placed back in the MPH where the mRNA solution was removed quantified and stored at -70°C until required.

2.7.5. Northern Analysis.

• Total RNA was loaded at 10-20µg/well and polyA RNA loaded at 3-6µg/well. Before loading, RNA volumes of approximately 5µl were mixed with 3 volumes (15µl) of RNA loading buffer (350µl formaldehyde, 1µl formamide, 150µl DNA loading buffer, 10µl ethidium bromide [stock 10mg/ml] and 200µl 5x MOPS buffer containing 0.1M MOPS, 40mM CH_3COONa and 5mM EDTA, pH7.0) and heated for 15 mins at 65°C.

• The samples were then loaded onto a 150ml flat-bed 1% (w/v) agarose gel containing 30ml of 5x MOPS buffer and 26.3ml of formaldehyde. The electrophoresis buffer was 1x MOPS buffer and this was recirculated during an 12hr run at 30V.

• Prior to blotting the gel was washed in dH_20 for 15 mins to remove the formaldehyde. RNA was transferred to nylon membrane (Hybond N+) by overnight capillary blotting (described by Sambrook *et al.*, 1989) using 20xSSC buffer. The RNA was UV-crosslinked using a UV Stratalinker 1800 and the positions of 18S and 28S ribosomal RNA marked. Hybridisation to specific probes was carried out as described in section 2.7.5.1.

2.7.5.1. Radiolabelling of cDNA probes.

For Northern blot analysis, $\alpha[^{32}P]$dCTP labelled DNA fragments were produced using the random priming technique. An Oligo-labelling kit (Pharmacia) was used to synthesise new cDNA strands in the presence of radioactively labelled dCTP.

• For each probe, 50-100ng of appropriate DNA, in a volume of 34µl with dH_20, was linearised by boiling and cooled on ice.

• 10µl of reaction buffer (containing random primer sequences, dATP, dGTP and dTTP, at the required salt concentrations) were added along with 5µl of $\alpha[^{32}P]$dCTP and 1µl of Klenow fragment. The reaction was incubated at 37°C for 1 hour.

• After incubation was complete, the unincorporated nucleotides were removed by centrifugation at 3000 rpm for 4 min through a Pharmacia spin column made of

Sephadex resin. The unincorporated nucleotides remained in the column while the labelled probe was collected in a clean Eppendorf.

• The degree of $\alpha[^{32}P]$dCTP incorporation was determined by analysis of 1µl aliquots in a Beckman LS5000CE counter and was 1-2 x 10^9 dpm/µg for each probe. The probe was boiled for 10 mins, placed on ice for 5 min and added to the hybridisation solution.

2.7.5.2. *Northern blots.*

• The nylon membranes were pre-hybridised at 42°C in Hybaid bottles or in plastic bags for 6-16 hrs in hybridisation buffer (5x SSPE, 10x Denhardt's, 2% SDS, 50% deionised formamide and 1mg/ml yeast RNA).

• Fresh hybridisation buffer was then added and the blot hybridised for 16 hrs with the relevant ^{32}P-labelled cDNA probe at 42°C.

After hybridisation, Northern blot membranes were washed as follows:-

• 2 x SSC / 0.1% SDS	4 washes of 15 min. each at room temperature
• 1 x SSC / 0.1% SDS	1 wash of 20 min at 65°C
• 0.1 x SSC / 0.1% SDS	1 wash of 15 min at 65°C

The membranes were then exposed to Kodak XAR-5 film for 1 to 7 days at -70°C, with an intensifying screen. After exposure the membranes were stripped by incubating them for 2hrs in a solution of 0.1% SDS at 65°C in a shaking waterbath. The membranes were reprobed with GAPDH cDNA probe, as a loading control.

2.8. Protein procedures.

2.8.1. Protein preparations from mammalian cells.

• Total cell lysates were prepared for Western blot analysis. 1ml of cell lysate (4ml 1M Tris pH6.8, 5ml glycerol, 25ml 10%SDS, 200mg BrdB, 16ml dH_20, 160µl of 2-Mercaptoethano) was added to a confluent 90mm plate of cells which were then homogenised using a cell scraper.

• The homogenate was transferred to a 1ml eppendorf and the DNA broken up by sonication, using a Fisons soniprep 150 MSE, for 30 seconds at 12 amplitude microns followed by a 5 minute spin at 12000g. At this point preparations were either stored at -20°C or boiled for 3 mins and loaded directly onto the gel (unless stated otherwise).

2.8.2. Liver Preparation for connexin 32 & 26 positive controls.

• The liver of one adult Spretus mouse was homogenised in 20mls of buffer (0.01% $NaHCO_3$). This homogenate was spun at 5000rpm for 15 mins in a Sorval ss34 rotor at 4°C.

• The pellet fraction was resuspended in 3 mls of dH_20 and added to a small 50 ml beaker containing 20g of Sucrose. A further 40 mls of dH_20 was added to the beaker and the sucrose allowed to dissolve.

• The sucrose solution was divided equally into two Beckman centrifuge tubes and 15mls of dH_20 added carefully to the top of the sucrose solution. This sucrose density gradient was spun for 1.5 hrs at 25000rpm.

• The resulting interface was carefully removed and spundown at 10000rpm for 15 mins in a sorval ss34 rotor. The pellet fraction was divided into 50ml aliquots and either stored at -20°C or loaded onto an 8% SDS-PAGE gel for Western blot analysis.

2.8.3. Polyacrylamide gel analysis of proteins.

Protein samples were resolved according to their molecular weight using sodium doecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE), in all instances 15cm gels were used. Polyacrylamide content varied according to the protein which was being resolved, (connexins 10%, cadherins and CAMs 8%). For a single 15cm gel the following solutions were prepared (two gels were made one to be used for Western analysis and the second to confirm equal loading.

Component volumes per 25 mls		Component solutions
8% gel	10% gel	
6.7ml	8.3ml	30% acrylamide/ 0.8% bisacrylamide
6.55ml	6.55ml	Running buffer (1.5M Tris, 4% SDS, pH8.9)
11.5ml	9.9ml	water
250µl	250µl	10% ammonium persulphate (freshly prepared)
15µl	12µl	TEMED

This resolving gel mix was poured between a sandwich of two glass plates sealed on three sides with a 0.75mm gasket. The solution was overlaid with isopropanol and left to polymerise at room temperature. Once the gel was set, the isopropanol was removed using Whatman 3MM filter paper and a 5% stacking gel solution added, together with the comb. The stacking gel was prepared as follows:

Component volumes per 10ml	Component
2ml	30% acrylamide/ 0.8% bisacrylamide
3ml	Stacking buffer (0.5M Tris, 4%SDS, pH6.7)
7ml	water
100µl	10% ammonium persulphate (freshly prepared)
10µl	TEMED

Once the stacking gel had polymerised the whole gel was transferred to an electrophoresis tank. The tank reservoirs were filled with Tris-glycine electrophoresis buffer (50mM Tris, 50mM glycine and 1% SDS in water). After removal of the combs, the wells were flushed out with electrophoresis buffer to remove any excess stacking buffer. Total cell lysates were added direct to the gel. To the liver preparations an equal volume of 2x SDS gel loading buffer, (4% SDS, 0.2% bromophenol blue, 20% glycerol, 100mM Tris, pH6.8 and 4% 2-Mercaptoethanol) was added. Once loaded the samples were resolved by electrophoresis at a constant current 30mA/gel for 2-3 hours. Once the dye front had reached the bottom of the gels, they were removed, one was stained for 3 hours in Coomassie blue (2.5g Coomassie brilliant blue, 100ml glacial acetic acid, 450ml methanol and 450ml dH_20), followed by incubation in destain (100ml glacial acetic acid, 450ml methanol, 450ml dH20) until protein bands were visible, in order to confirm equal loading. The second gel was used for Western analysis.

2.8.4. Western blotting analysis.

Protein samples (prepared as described in section 2.8.1) and resolved by SDS-PAGE (section 2.8.3).

- After removal from the tank, the gel was trimmed and measured. Six pieces of Whatman 3MM filter paper and 1 piece of Nitrocellulose membrane (ECL-hybond) were cut to an equal size. Transfer of proteins from the gel to the Nitrocellulose was performed on a semi-dry electroblotter.
- 3 pieces of pre-sized filter paper were soaked in the transfer buffer and placed on the semi-dry blotter.
- The nitrocellulose membrane was soaked in dH_20 and placed on top of the gel. The gel and membrane were then placed on top of the filter papers with the gel uppermost.
- 3 more pieces of buffer soaked filter paper were placed on the gel, the lid replaced and electroblotting performed at a current of Amps for 1hr 30 mins.
- Once the transfer was complete, the nitrocellulose membrane was placed in blotto, (blocking solution: 5% Marvel in PBS) and shaken for 1 hour at room temperature.

The membrane was then incubated overnight at 4°C with the primary antibody diluted in blotto to the appropriate concentration (see figure legends).

• This was followed by 3 washes with blotto for a duration of 20, 15 and 10 mins respectively. The horseradish peroxidase secondary antibody was diluted in blotto at 1:1000 and incubated with the membrane for 1 hour at room temperature.

• After a quick rinse with PBS the membrane received two 5 minute washes in blotto followed by a further two 5 minute washes in TBS-Tween (100mMTris pH 7.5, 0.9% NaCl, 0.1% Tween in a final volume of 100mls). Chemiluminescence was used to detect signals.

To develop the blots, an Amersham, *Enhanced chemilluminescence (ECL)* kit was used, according to manufacturers instructions. 10mls of Solutions 1 and 2 were mixed in a small beaker, added to a box containing the membrane and incubated for 1-2 mins. The solution was poured away and excess liquid was removed. The membrane was quickly wrapped in Saran wrap and exposed to Fuji RX film for 5 seconds to 60 mins, (depending on the strength of the signal).

2.8.5. Immunofluoresence

• Cells were grown until 80% confluent on single well glass chamber slides. After removal of tissue culture media the cells were washed once with PBS and fixed in ice-cold acetone for 30 mins.

• The acetone was removed and a 1.5% Marvel / PBS blocking solution applied to the cells for 10 mins. After removing the blocking solution 50-100µl of primary antibody was applied at the appropriate concentration (see figure legends) and left in a moist chamber at room temperature for 1-2 hours.

• The cells were then washed 3 times with the blocking solution and incubated with 50-100µl of secondary FITC-conjugated antibody for 1 hour. The cells received a final wash in blocking solution and were then mounted in 50% glycerol in PBS. Cells were analysed under UV illumination using a Leitz vario orthomat microscope.

CHAPTER THREE

RESULTS

3.1. Introduction

The aberrant growth of certain transformed cells can be suppressed when cultured in the presence of excess resting normal cells (Stoker 1967; section 1.6). This phenomenon has been correlated with the presence of heterologous gap junctional communication between the two cell types (Yamasaki and Katoh, 1988). The focus formation assay, which has been the major analytical tool for studying the phenomenon, typically consists of a co-culture of normal and transformed cells seeded at 10^6 and 500 cells per dish respectively. After a period of 7-14 days the number of foci are counted and measured, compared to the control (separate cultures of 500 transformed cells) and the extent of inhibition calculated. In its present form the focus assay can only be used to study those cells that escape inhibition; single cells or small, possibly slow growing, colonies cannot be identified. The information that can be gained from this assay is limited to determining whether suppression occurs. As a result investigators have concentrated on the role of junctional communication in the inhibition phenomenon and neglected to examine, in any detail, the phenomenon itself. There are several important questions regarding this mode of suppression which remain to be answered:

1. Is there a threshold colony size, above which the transformed cells maintain their aberrant growth phenotypes?
2. In large foci is the inhibition greatest at the focus periphery rather than in the centre, as might be expected if GJIC is the pathway of suppression?
3. Several reports have shown that even where there is no inhibition of focus number there is often inhibition of focus size. Is this due to compression of the foci by the physical presence of the normal cells or to a reduction in growth rate relative to the controls?
4. Do transformed cells respond in different ways when different normal cells are present?
5. Do the transformed cells stimulate normal cells to divide?
6. Is the growth of the transformed cells sometimes stimulated by the presence of the normal cells (possibly by a feeder effect)?

3.1.1. Experimental system and selection of cell lines for study.

For this project a new assay has been developed which will facilitate the study of suppression in greater detail. A lineage marker, β-galactosidase has been introduced into the transformed cells to enable them to be unambiguously identified and counted in co-culture. The proliferative status of normal and transformed cells has been determined using a growth assay based on [^{3}H]-thymidine incorporation and analysis by autoradiography. This has allowed the study of cell-cell interactions which lead to changes in growth, to be assessed by analysis of individual cells. Answers to questions

such as those listed above should provide more information on a phenomenon that may play an important role in primary defence against cancer (Section 1.6).

In order to examine the inhibition in detail, cell lines were required which showed the phenomenon described by Stoker. Ideally, a set should be included that contains a tumour cell type that is poorly coupled, produces foci on a background of normal cells and upon genetic modification to correct the defect in junctional communication show an increase in junctional communication and inhibition of focus formation. In previous studies many investigators have used phorbol-esters (usually TPA) and growth regulators such as cAMP as a means of manipulating the levels of gap junctional communication between cells. However, these chemicals act only transiently and are not specific modulators of GJIC; their mode of action on this pathway is also poorly understood. The genetic manipulation of protein expression to stably regulate GJIC provides a more specific means of analysis. Appropriate cell lines were obtained from Prof. F. Tato (Rome).

The tumour cell line S180 is poorly coupled (Musil et al 1990, Prowse 1992) and forms foci in the presence of excess, growth inhibited normal Rat2 cells (Tato, personal communication). However, upon transfection with a cDNA for E or N cadherin their focus forming ability is markedly reduced (Tato personal communication) and it might be expected, on the basis of work by Musil et al (1990; section 1.4.6) that these cadherin transfected cells (S180E217 - E cadherin transfectant, S180NII & S180N2A1 - N cadherin transfectants) should show increased levels of GJIC. In addition to Rat2, an E cadherin transfected Rat2 clone (R24E) was available that was reported to have an elevated level of GJIC (Tato, personal communication). This will provide an opportunity to examine if changes in the communication phenotype of the normal cell effect the inhibition phenomenon.

The low level of homologous communication observed in cultures of S180 cells is typical of many tumour cell types and it is often suggested that the reduced coupling contributes to the escape from normal growth control (section 1.4.6). However, tumourigenesis is not always associated with loss of coupling (section 1.4.6), so it is unlikely that all well coupled tumourigenic or transformed cells are suppressed. Such cells may have partially, or completely, lost the ability to respond to inhibitory growth signals that use the junctional pathway. Analysis of transformed cell lines that are coupled to normal cell lines, but are not inhibited by them, should provide a useful comparison, as well as a control for focus inhibition caused by other factors such as cell crowding or nutrient deprivation. Therefore, focus forming cell lines which show high levels of communication have been included in this study. With these cell lines even low levels of growth inhibition should be detectable using the more sensitive assays developed for this project. Furthermore, analysis of uninhibited transformed cells lines can be used, together with the S180 cells, to examine whether normal cells are stimulated by proliferating transformed cells.

To obtain cell lines with these characteristics the communication phenotypes of a range of transformed cells was surveyed. Two were selected; BICR, a rat mammary carcinoma cell line that is very well coupled relative to S180, and SVT2, a 3T3-SV40 transformed cell line that shows intermediate levels of communication and high tumourigenicity (tumours are produced within 5-7 days of a subcutaneous injection, Fernandez et al 1992). The results are presented in the following section.

The focus forming ability of the selected cells was then examined in the presence of normal Rat2 and 10T1/2 cells. The 10T1/2 cell line has been used extensively in previous studies that examined the focus forming potential of 10T1/2 transformed cells (Mehta et al 1986 & 1991). Here the effects of 10T1/2 cells on unrelated transformed cell lines are examined. Furthermore, the inclusion of a second normal cell line in this study provides an opportunity to examine whether different normal cells have similar abilities to inhibit the growth of specific transformed cells.

The Rat2 cells used in this study have a low level of thymidine kinase and therefore incorporate ^{3}H-thymidine at low levels. This leads to a reduced, but detectable grain count above those cells that are going through S-phase, when analysed by autoradiography. However, this should provide a convenient reporter for GJIC as dividing Rat2 cells in co-culture with dividing transformed cells will have higher grain counts when the two cell types metabolically co-operate.

The following results chapter has been divided into 3 parts. Part 1 deals with the characterisation of the growth and communication phenotypes of the different cell lines. Part 2 examines the focus forming ability of the various transformed cells on different backgrounds of normal cells. Part 3 describes the new assay system, developed for this project, and its application to the study of growth inhibition in the cell lines characterised.

3.2. THE GROWTH AND COMMUNICATION PHENOTYPES OF THE CELL LINES.

3.2.1. Growth phenotypes of cell lines under study.

For this, study growth inhibition of the transformed cells is contingent on the presence of excess, growth inhibited normal cells. Therefore conditions must be established which markedly reduce the growth of normal cells, but not the growth of transformed cells in separate cultures. The two methods commonly used are high cell density or serum deprivation. During preliminary experiments it was found that growth inhibition of Rat2 and 10T1/2 cells could be achieved more reproducibly by growing cultures to saturation density (SatD) rather than by reducing the serum concentration in the media to 0.5%. The saturation density of all of the cell lines was examined using a proliferation assay based on [^{3}H]-thymidine incorporation and analysis by autoradiography.

Separate cultures of each of the cell lines were set up at $5x10^5$ cells / 90mm dish, in DMEM10% (DMEM growth medium supplemented with 10% FCS). Cell counts were then taken every 12 hours, using a haemocytometer, to measure total cell number. Trypan blue staining was used to measure cell viability (section 2.2.2). Growth curves were plotted from which the population doubling time (PDT) and terminal density (TD) were determined. TD refers to the stauration density of the normal cells and the density at which the transformed cells begin to die due, for example, to high pH. The β-gal transfected cell lines were also analysed to ensure the exogenous gene did not alter their growth characteristics.

The labelling index (proportion of cells incorporating [^{3}H]-thymidine) was also measured in order to determine the proliferative activity of the cell population. Cultures were set up at $5x10^5$ cells / 90mm dish in DMEM10% and analysed every 24 hours. Prior to fixation (in 1% formaldehyde and 0.1% gluteraldehyde in PBS for 30 mins) the cells were pulsed for 18 hours with [^{3}H]-thymidine (0.185MBq/ml). The cultures were then analysed using autoradiography (section 2.5.2). Growth parameters for each cell line are shown in Table 3.1.

Table 3.1. Growth parameters of cell lines.

Cell lines	TD $x10^{-6}$			PDT (hours)
	β-gal $^-$	β-gal$^+$		
Rat2	4.5 (1.1)	4.5 (0.8)		19 (3.3)
R24E	4.9 (0.6)	4.8 (1.1)		20 (2.8)
10T1/2	4.4 (0.6)	5.0 (1.0)		25 (4.8)
S180	8.2 (1.1)	7.1 (1.5)	*	14 (2.0)
S180N2AI	10 (2.4)	10 (1.8)	*	16 (1.6)
S180NII	7.6 (0.8)	7.3 (1.5)	*	13 (3.5)
S180E217	6.7 (1.1)	7.8 (2.1)	*	14 (2.4)
SVT2	11 (1.8)	10 (1.7)	*	17 (4.2)
BICR	4.5 (0.5)	5.0 (0.8)	‡	13 (1.5)

Continued over page.

Table 3.1. Continued.

Cell lines	LI (%) at 24 hrs β-gal $^-$	β-gal $^+$	LI (%) at 48 hrs β-gal $^-$	β-gal $^+$	LI(%) at TD β-gal $^-$	β-gal $^+$
Rat2	100	100	4.5 (0.7)	5.2 (2.4)	2.4 (0.4)	3.5 (1.5)
R24E	100	100	4.8 (0.5)	4.0 (1.8)	2.7 (0.2)	2.5 (0.4)
10T1/2	100	100	8.2 (1.0)	10 (3.5)	4.8 (0.6)	4.0 (1.0)
S180	100	100	75 (3.5)	68 (5.4)	43 (6.5)*	38 (1.5)*
S180N2AI	100	100	60 (5.0)	66 (7.0)	45 (9.3)*	52 (5.5)*
S180NII	100	100	67 (3.0)	54 (6.0)	36 (5.2)*	30 (4.3)*
S180E217	100	100	58 (4.8)	77 (5.4)	32 (3.3)*	35 (7.8)*
SVT2	100	100	80 (6.0)	67 (9.0)	47 (6.8)*	52 (8.9)*
BICR	100	100	50 (2.5)	48 (3.5)	‡	‡

Table 3.1. To obtain the population doubling time (PDT) and terminal density (TD) cultures were plated at 5 x 10^5 cells / 90mm dish in DMEM10%. Total cell counts were taken every 12 hours. For the proliferation analysis, cultures of each cell line were seeded at 5 x 10^5 cells / 90mm dish in DMEM10% and analysed every 24 hours. 18 hours prior to fixation cultures pulsed with [^{3}H]-thymidine (0.185MBq/ml) fixed in 1% formaldehyde and 0.1% gluteraldehyde in PBS for 30 mins. Cultures were then processed for autoradiography according to the method given in section 2.5. LI - Labelling Index. Standard deviations are given in parentheses. Data provided are averages from at least three replicate cultures. PDT values were not significantly different for β-gal$^+$ and β-gal$^-$ cell lines (P>5%). (*) Indicates cultures where cells continued to grow at confluence and began to multi-layer and often detach. (‡) Indicates that BICR cells began to dye soon after reaching confluence, attempts were made to obtain the LI at confluence but the majority of cells detached during fixation.

The LI's for the Rat2 and 10T1/2 cells drop from 100% at 24hrs to 4.5% and 10%, respectively, after 48 hours. This drops further, to 2.4% and 4.8% upon reaching their respective terminal densities. The transformed cells maintain higher LI's (between 48% - 80% after 48 hrs and between 30% and 52% at the time when the normal cells reach saturation density). The transformed cell lines did not reach a defined saturation density but continued to grow, multi-layer and detach. Student t-tests carried out on the growth parameters obtained from the β-gal$^+$ and β-gal$^-$ cell lines indicates that the expression of the transfected β-gal gene in the transformed cells has no significant effect on their growth phenotypes (in all instances P>5%). Transfection of E or N cadherin into the S180 cells does not have a significant effect on the growth parameters measured here (P>5%). On the basis of these results the various cell lines provide the opportunity to examine whether, in co-culture, the normal cells, upon reaching high cell density, are capable of suppressing the growth of the transformed cells.

3.2.2. Communication Phenotypes.

3.2.2.1. *Homologous communication.*

The selected cell lines were examined for levels of homologous communication. Levels of communication in both β-gal$^+$ and β-gal$^-$ cell lines were compared to ensure the exogenous gene had no effect on their communication phenotypes. The levels of homologous communication which were recorded (Table 3.2), were obtained using two techniques; nucleotide transfer and dye transfer. The technique of nucleotide transfer is based on the transfer of [^{3}H]-nucleotides (derived from ^{3}H-uridine) through gap junctions. The labelled nucleotides are incorporated into the RNA of coupled cells and the extent of transfer can be analysed using autoradiography. The technique has advantages over dye transfer in that the cells incur no physical damage, as is the case in micro-injection, and the principle behind the technique has a direct relevance to biological events i.e. the exchange of metabolites over a long time course (4 hours rather than the 1-2 mins used for the analysis of dye transfer). Furthermore, it can be easily applied to determine levels of heterologous communication, as the donor cells can be intrinsically distinguished from the recipient cells by the larger grain counts over the donors, due to the preparative labelling period (Pitts & Simms 1977). However, dye transfer is more widely used and has, therefore, been carried out also, to see if the two techniques generate comparable data (for methods see section 2.3). Representative nucleotide and dye spreads are illustrated in Figure 3.2 and the average levels of homologous communication recorded for each cell line, are given in Table 3.2.

The two techniques appear to generate comparable data. For example, S180 and BICR cells are, respectively, the poorest and best coupled as determined by both methods of analysis. In the majority of instances nucleotide transfer is the more sensitive of the two techniques; this is not surprising given that transfer and incorporation of nucleotides occurs over 4 hours rather than the 2 mins allowed for dye transfer (a time chosen that generates large enough differences between coupled and uncoupled cells whilst limiting cell damage). Attempts to determine levels of communication for BICR cells using nucleotide transfer were made difficult by the fact that some of the cells were lost during fixation (see Figure 3.2.b.iii), leading to a decreased count. Fixation did not effect the other cell lines in this way.

Both Rat2 and 10T1/2 cell lines show levels of communication typical of many fibroblasts. The transformed cells, SVT2 and BICR, show comparable levels of communication with the normal cells, suggesting, that in these instances, homologous communication is a poor indicator of cell tumourigenicity.

S180 cells show a low level of communication which is typical of many tumour derived cell lines (Kalimi et al 1992). Transfection of the S180 cells with a cDNA for E or N cadherin increases the level of communication markedly. This is associated with a distinct change in cell shape to a flatter, fibroblast-like morphology, (Figure 3.2.cii & dii). In contrast to the S180 E & N cadherin transfectants, R24E cells (Rat2 E cadherin transfected cell line) show a significant reduction ($P<1\%$) in homologous transfer in comparison to the Rat2 parental cell line. Two other clones of Rat2 E cadherin transfected cells were examined, R2013E and R206E, their respective homologous communication levels (as determined by dye transfer) were 8.8 (sd 3.9) and 13 (sd 4.7) respectively. Although the E cadherin expression appears to decrease the level of homologous communication in these cell lines, no apparent difference in morphology was observed between these cells and the parental Rat2 cell line.

Table 3.2. Homologous GJIC.

CELL LINE	DYE TRANSFER β–gal $^-$	DYE TRANSFER β–gal$^+$	NUCLEOTIDE TRANSFER β–gal$^+$
Rat 2	19.4 (6.7)	16.8 (4.9)	32.3 (12)
R24E	12.5 (6.4)	12.0 (2.5)	19.5 (8.7)
10T1/2	10.6 (4.4)	9.3 (1.2)	22.4 (8.4)
S180	1.6 (1.3)	2.7 (2.0)	2.2 (1.5)
S180E217	12.0 (3.1)	13.3 (4.2)	20.3 (5.5)
S180N2A1	13.4 (3.7)	12.3 (5.0)	17.7 (6.5)
S180NII	18.6 (7.7)	20.8 (8.5)	15.8 (6.6)
SVT2	25.3 (8.9)	23.1 (4.5)	19.6 (3.2)
BICR	41.5 (9.5)	47.5 (11)	34.7 (8.6)

Table 3.2. The proportion of injected or donor cells which showed transfer to at least one surrounding cell (frequency of transfer) was 100% for the majority of cell lines with the exception of S180 where the frequency of transfer was 60%. For dye injection, individual cells were injected ionophoretically with Lucifer Yellow CH for 2 minutes and the number of cells into which dye had spread were counted. The mean dye spreads shown are calculated from a minimum of 30 injections (10-20 injections per dish, 2-3 replicate dishes) and the mean nucleotide spreads calculated from the analysis of 30-50 donor cells, (10 donors per slide, 3-5 slides per cell line). Donor cells were prepared by growing cells to 70% confluence, labelling for 4 hours with [^{3}H]-uridine (0.46 MBq/ml), then washing 4 times with unlabelled medium. After trypsinisation 100-500 donor cells were added to 80-100% confluent monolayers of recipient cells. After 4 hours the co-cultures were washed 3 times in PBS and fixed in formal saline (0.9% NaCl, 10% formaldehyde in PBS) for 30 mins. They were then extracted with TCA and processed for autoradiography according to the method given in section 2.5. Background levels of [^{3}H]-uridine were determined by counting the number of grains over a minimum of 50 cells which were situated away from any donor cells and the average grain count per cell determined. Transfer was recorded when grain counts directly above the cells were greater than twice the background. Standard deviations are given in parentheses. The difference between the values obtained from the β-gal$^+$ and β-gal$^-$ cell lines is not significant (P>5%) for all of the cell lines.

Figure 3.2. Examples of homologous dye and nucleotide transfer.

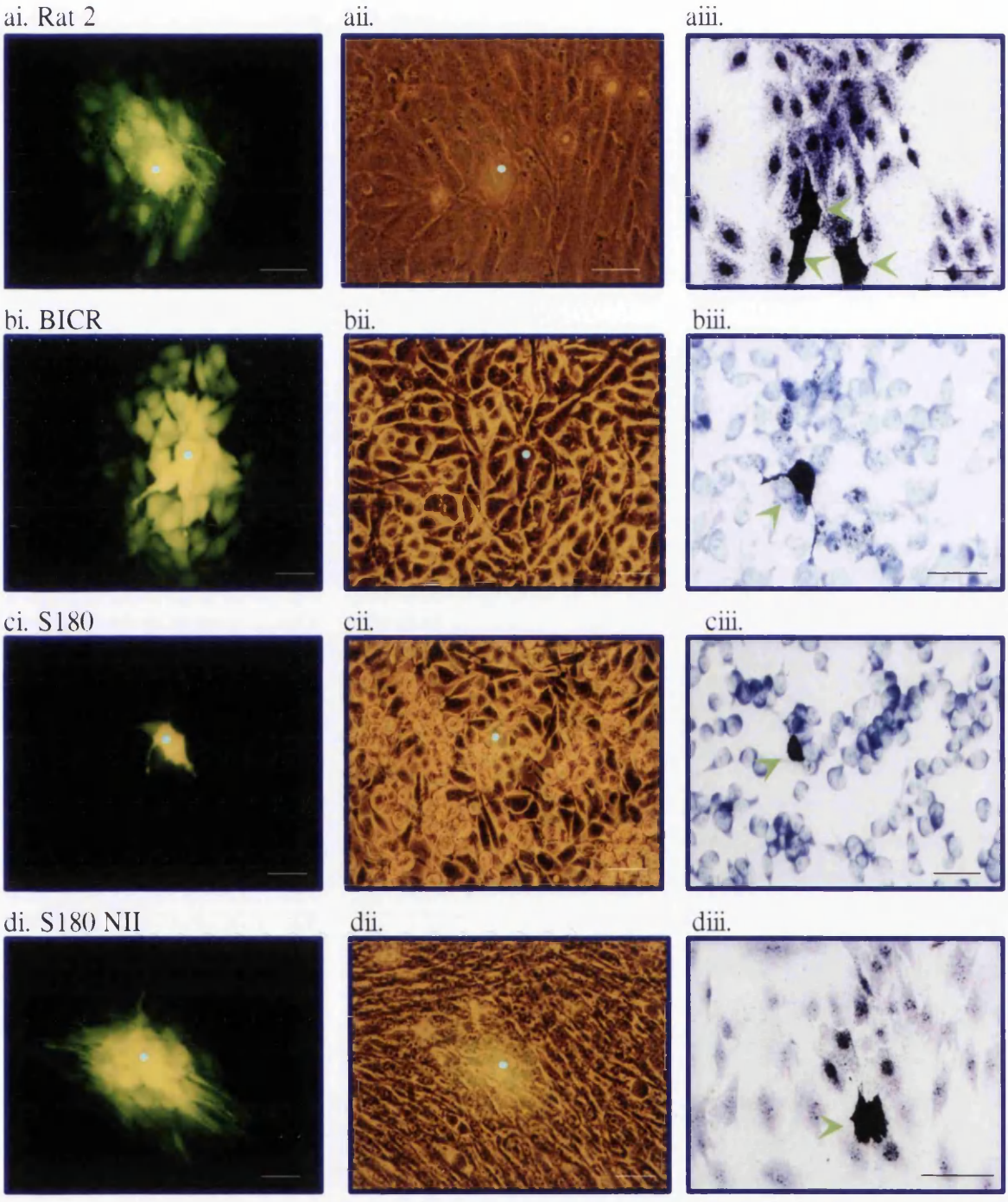


Figure 3.2. Photomicrographs of dye spreads, after microinjection of Lucifer Yellow CH, and of nucleotide transfer. Blue dot indicates cell injected with tracer dye. See Table 3.2. legend for experimental details. Figures ai-di are fluorescence images of the phase contrast micrographs shown in aii-dii.
Nucleotide transfer micrographs are shown in Figures aiii-diii. Cell lines correspond to those shown in ai-di. Green arrows indicate donor cells. Bar 50μm.

3.2.2.2. *Heterologous communication between normal and transformed cells.*

Attempts were made to use dye transfer as a means of examining heterologous communication. In order to distinguish the two cell types in co-culture the transformed cells were first labelled with fluorescent microspheres (separate cultures of transformed cells were incubated for 5hrs with 4μl of the microsphere suspension supplied by Cambridge Bioscience - section 2.1.2). Cultures were then washed 4 times with PBS, trypsinised and approximately 1000 labelled cells were added to 80% confluent cultures of normal cells and left to attach for 4 hours.

However, during the dye transfer assays it became clear that the fluorescent glow from the microspheres was the same colour and in some instances the same intensity as the Lucifer Yellow CH tracer dye. Ambiguity arose when measuring dye transfer, particularly when the dye spreads were faint. Thus it was not always possible to determine with confidence when the dye had transferred into a labelled cell or whether the glow of the microspheres was responsible for the fluorescence observed - see Figure 3.3. Furthermore in some instances beads which had not been endocytosed by the transformed cells but were loosely attached to the cell surface, often detached when transferred to the co-culture, leaving the possibility that they could become endocytosed by the normal cell line leading to false identification. The technical problems associated with this technique made nucleotide transfer the preferred assay.

The experimental procedure for examining heterologous communication using nucleotide transfer is the same as that used to measure homologous communication (section 3.2.2.1), however, in this instance the donor cells were the transformed cells and the recipient cells were the normal cells.

Due to specificity of gap junction formation (section 1.4.5.2) two cell lines that each form homologous gap junctions may not necessarily form heterologous gap junctions. For example, communication is often restricted between epithelial cells and fibroblasts (section 1.4.5.2). The efficiency with which heterologous gap junctions form between the cell combinations examined here, varies in terms of the frequency and the level of nucleotide transfer. Average nucleotide transfers obtained for the cell combinations examined are provided in Table 3.3 and representative spreads are shown in Figure 3.4

In terms of levels of heterologous communication, R24E cells form the best normal cell partner with the transformed cell lines followed by Rat2 and 10T1/2 cells. However, the order (ranked by level of heterologous communication) in which the transformed cells communicate with the normal cells is generally the same regardless of which normal cell line is used. For example, BICR cells show the highest level of coupling to the normal cells, regardless of which normal cell line is the heterologous partner, and S180 cells show the lowest coupling to the normal cells.

Figure 3.3. Analysis of heterologous communication using fluorescent beads as lineage markers.

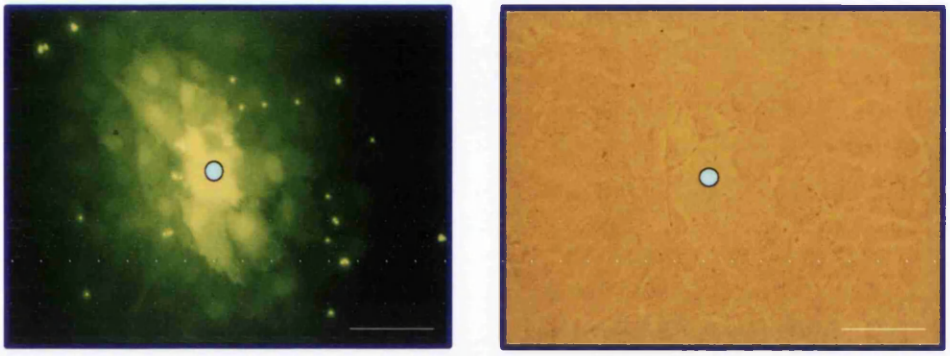


Figure 3.3. 80% confluent cultures of SVT2 cells were labelled with 4 µl of microsphere suspension (supplied by Cambridge Bioscience; section 2.1.2) for 5hrs. These cultures were then washed 4 times with fresh medium, trypsinised and approximately 1000 cells added to 80% confluent cultures of unlabelled Rat2 cells. Co-cultures were left for 4 hours prior to dye injection analysis. In the above micrographs a Rat2 cell has been injected with Lucifer Yellow for 2 mins (injected cell indicated by blue dot). However, the fluorescent halo, which can be seen around the beads in the above figure, could be misinterpreted as faint dye spread, particularly when dye spreads in the surrounding cells were also faint. Furthermore, beads which were attached to the surface of the cells (but not endocytosed) often to detached in co-culture and the possibility exists that they could be incorporated by unlabelled cells. Bar 50 µm.

BICR cells are very well coupled to Rat2 & R24E cells whereas S180 & S180E217 cells are poorly coupled to these cells. The remaining transformed cell lines show intermediate levels of communication with Rat2 & R24E cells (relative to BICR and S180 cells). All transformed cells communicate relatively poorly with 10T1/2 cells and no detectable transfer is observed when S180 cells are the heterologous partner. In all instances the S180 E and N cadherin transfected cells show increased heterologous communication relative to the S180 parental cells.

An example of gap junction specificity is illustrated here by the S180E217 cells which are well coupled to each other (Table 3.2) but are poorly coupled to the normal cells (Table 3.3). E cadherin tends to confer an epithelial phenotype on the cells which express it (Musil et al 1991) and the change in morphology of the S180E217 cells would tend to support this. However, these cells were relatively poorly coupled to R24E cells, which have also been transfected with E cadherin. This would suggest that E cadherin expression in both cell types is insufficient to ensure heterologous gap junction formation. However, R24E cells maintained a fibroblast morphology and an apparent decrease in homologous communication, and the possibility exists that although transfected with a E cadherin cDNA they do not express the protein. Protein expression has been examined in section 3.2.3.

It is not clear why the N cadherin transfected S180 cells show high levels of communication with the Rat2 cell lines yet are poorly coupled to the 10T1/2 cells. Studies have been carried out in the following section in order to examine the expression of specific adhesion and connexin proteins which may mediate the phenomenon of gap junction specificity seen here.

Table 3.3. Heterologous communication.

CELL LINE	Rat 2		R24E		10T1/2	
	level	freq. %	level	freq. %	level	freq. %
S180	0.47 (0.2)	23	1.07 (0.3)	40	0.00	0
S180E217	2.0 (1.5)	37	4.80 (1.9)	77	2.0 (1.4)	53
S180N2A1	12.3 (4.3)	100	14.8 (5.0)	100	3.70 (1.6)	70
S180NII	6.10 (2.7)	87	14.7 (4.7)	100	0.9 (0.5)	43
SVT2	5.73 (3.8)	80	8.30 (2.6)	97	2.33 (1.3)	33.3
BICR	15.9 (2.2)	100	29.9 (9.5)	100	4.57 (3.2)	90

Table 3.3. For experimental details see section 3.2.2.1 & legend for Table 3.2. The mean level of transfer for each cell combination was calculated from the analysis of 30-50 donor cells, (10 donors per slide, 3-5 slides per cell line). The frequency (freq.) of transfer (proportion of donor cells which showed transfer to at least one surrounding cell) is given as a percentage. Standard deviations are given in parentheses.

Figure 3.4. Examples of heterologous nucleotide transfer spreads.

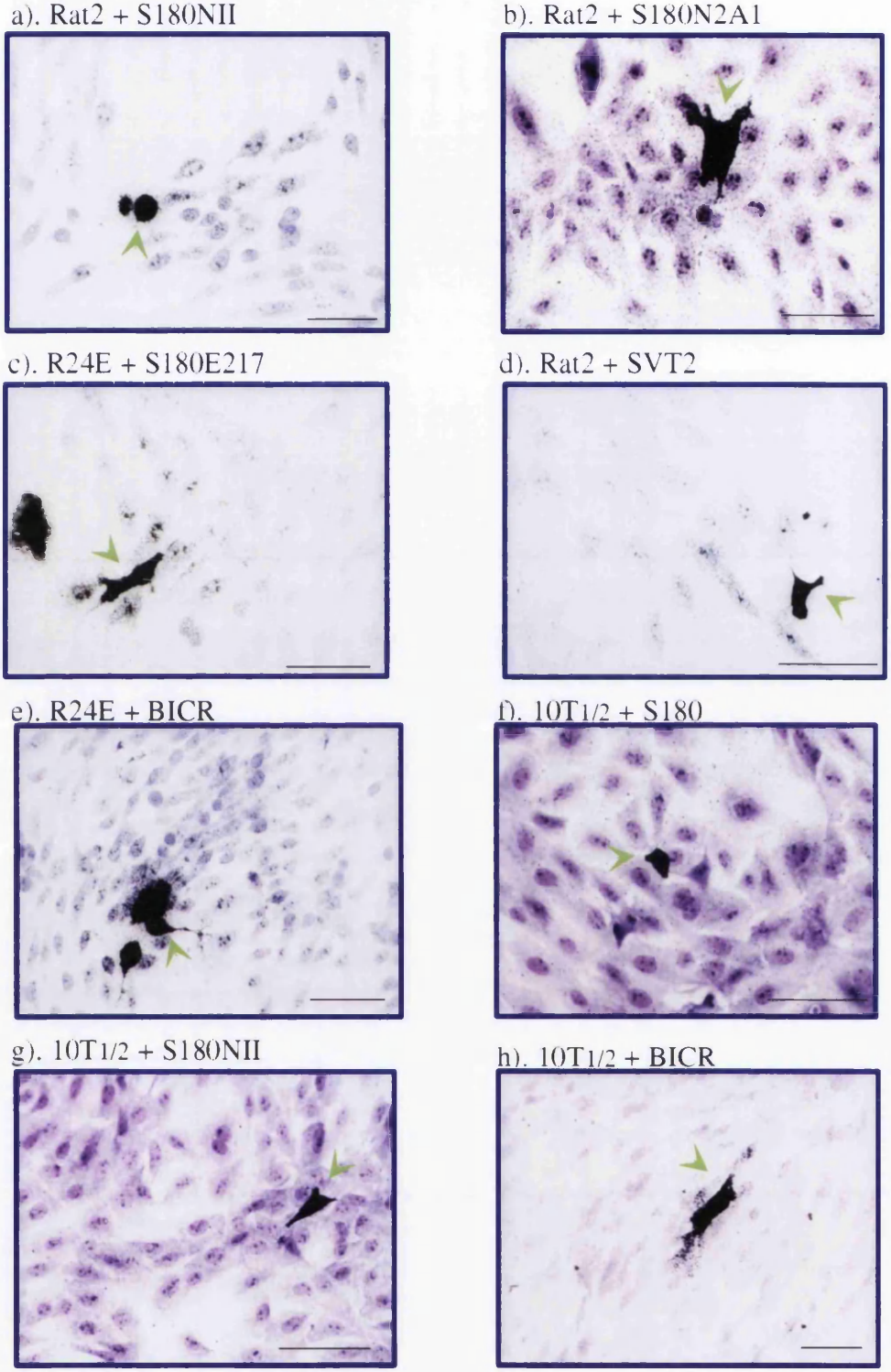


Figure 3.4. a-h show examples of nucleotide transfer between various normal and transformed cells in contact (cell combinations are given above each figure). Donor cells (transformed cells) are indicated by green arrows. See Table legend 3.3 & section 2.3.2. for experimental procedure. The above cultures were over-exposed to enhance visualisation. Bar 50 µm.

3.2.3. Expression of gap junction associated proteins.

It has been suggested that communication compartments *in vivo* are a manifestation of specificity of gap junction formation (section 1.4.5.1). It has been observed that the cells of such compartments follow different lineage pathways from cells of different compartments (Kam & Hodgins 1992; section 1.4.5.1). In culture, specificity of gap junction formation has been reported between a variety of transformed and normal cell lines (levels of heterologous communication are often very low or zero; Kalami et al 1992). This is thought to arise due to a defect in either adhesion-mediated cell-cell recognition or the loss or modification of gap junction associated proteins (section 1.4.6). Restoration of the heterologous junctional pathway can result in the growth inhibition of the transformed cells (Mehta et al 1991; section 1.6).

The molecular basis of gap junction specificity is, however, not clearly understood. In an attempt to explain the observed patterns of heterologous communication among the cell lines in this study, expression levels of the following adhesion molecules have been examined; E cadherin, N cadherin and NCAM, together with the expression of the gap junction associated protein - connexin43 (Cx43).

Western blot analysis was used to detect level of expression and immunofluorescence was employed to localise protein expression within the cell.

3.2.3.1 *Connexin43 expression.*

Although the evidence is mainly correlative connexins have been widely reported as playing an essential role in the formation and regulation of gap junctions (section 1.3.1.2). Cx43 is the most widespread connexin and is expressed by a wide variety of vertebrate cell lines (Beyer et al 1989, Rogers et al 1990). It has been shown that cells which previously expressed other connexin types *in vivo,* express Cx43 in culture (Stutenkemper et al 1992). Cx43 is thought to be synthesised as an unphosphorylated 43kDa species (Cx43-NP). However, many cell lines which are well coupled, also express a phosphorylated, 47kDa species (Cx43-P_2; Saez et al 1986, Musil et al 1990, Musil & Goodenough 1991). Intermediate forms (44-46 kDa; Cx43-P_1) have also been observed (Musil et al 1990 & 1991). Figure 3.5.a. shows the results of Western blot analysis using a rabbit monoclonal antibody against Cx43 (section 2.1.5). Equal loading of samples was confirmed by Coomassie blue staining of replicate gels.

Cx43-P_2 is observed in all of the cell lines examined here, although the level of protein expression, as indicated by the intensity of the band, varies between the different cell lines (Figure 3.5.a). In the S180 set of cell lines, S180-parental cells predominantly express Cx43-NP and only low levels of Cx43-P_2. After these cells are transfected with cDNA for E cadherin, the expression level of Cx43-P_2 increases. This correlates with an increase in homologous communication (Table 3.2) and confirms reports published by Musil et al (1990) who showed that E cadherin expression in S180 cells results in

increased conversion of Cx43-NP to Cx43-P_2 and subsequently higher levels of communication. Results from the study carried out here also show that expression of N cadherin in S180 cells results in increased expression of Cx43-P_2 and increased communication. It is not known whether cadherins directly regulate GJIC or do so via the regulation of Cx43-P_2.

Rat2 parental cells predominantly express Cx43-P_1 and only relatively low levels of Cx43-P_2. 10T1/2 cells express very little Cx43 (of any form), yet both of these cell lines form gap junctions very efficiently (Table 3.2). Transfection of the Rat2 cells with E cadherin appears to increase the expression of Cx43 (phosphorylated and unphosphorylated). This is consistent with the data from the S180 series and would suggest that cadherins can regulate the expression of Cx43. The increased levels of Cx43-P_2 expression in R24E cells are not, however, associated with a increase in homologous communication within this cell line (Table 3.2).

High levels of Cx43 are expressed in BICR cells which is consistent with the finding of others (Prowse 1992).

It has been suggested that discrete, punctate Cx43 immunostaining at points of cell-cell contact is associated with EM-identifiable gap junction structures (Beyer et al 1989, Dermietzel et al 1989, Musil et al 1990). Immunofluoresence analysis was used to examine whether Cx43 distribution relates to the communication phenotypes off the various cell lines examined.

BICR cells showed the greatest amount of immunostaining predominantly cytoplasmic and perinuclear but also at points of cell-cell contact (Figure 3.5.b) and this is consistent with the high expression levels of this protein as indicated by Western blotting analysis. S180 cells show no detectable Cx43 immunostaining (Figures 3.5.c). However, the detection of Cx43 in these cells by Western blotting analysis would suggest that the immunofluoresence assay is not sufficiently sensitive or that the protein is masked and inaccessible to the antibody. This data does not support that of Musil (1991) who found that S180 cells do show very low levels of immunostaining although only at perinuclear sites. The S180 E and N cadherin transfectants do show discrete, punctate Cx43 immunostaining at points of cell-cell contact (Figure 3.5.d. & e). It is not clear which Cx43 species is responsible for this punctate staining as the antibody does not distinguish between the phosphorylated and unphosphorylated forms. Reports by Musil et al (1992) suggest that it is Cx43-P_2, as they found that Cx43-NP distribution in S180 cells was limited to perinuclear staining and conversion to Cx43-P_2 correlated to a punctate staining pattern at cell contact points, which in turn would suggest that Cx43 phosphorylation is involved in gap junction formation. R24E cells show greater levels of Cx43 immunostaining than Rat2 or 10T1/2 cells. In all 3 cell lines the protein is localised throughout the cells in general, including points of cell-cell contact.

The expression of Cx26 and Cx32 was examined using appropriate rabbit monoclonal antibodies (section 2.1.5). None of the cell lines examined appear to express Cx32 or Cx26. Data not shown.

3.2.3.2. *Cadherin expression.*

Western blotting analysis revealed no endogenous E cadherin expression in S180 and Rat2 parental cells, SVT2, BICR or 10T1/2 cells. However, expression is observed in the cell lines transfected with E cadherin cDNA (S180E217 and R24E cells), together with the epidermal positive control cell line - P6 (Figure 3.6.a). S180E217 cells appear to express the highest amount of the 120kDa cell-cell adhesion protein. Immunofluorescence analysis reveals that the protein, in R24E and S180E217 cells is distributed along areas of cell-to-cell contact (see Figures 3.6.b & c). S180E217 cells show higher levels of immunostaining than R24E cells which is consistent with the Western blot data.

The group that originally carried out the transfections confirmed that the cell lines, S180NII and S180N2A1 both expressed N cadherin at the Western level and immunofluorescence analysis localised the protein to areas of cell-cell contact. In addition, S180, Rat2 and R24E cells, do not express N cadherin (Prof. F. Tato - personal communication). 10T1/2 and SVT2 cells do not express N cadherin - data not shown. BICR cells have previously been shown to express N cadherin at areas of cell-cell contact (Prowse 1993).

Using Western blotting analysis it was shown that none of the cell lines examined here expressed P cadherin - data not shown.

3.2.3.3. *NCAM expression.*

Expression studies using Western analysis, (Figure 3.7.a) reveal that the normal cell lines, Rat2, 10T1/2 and R24E and the transformed cell line SVT2 express NCAM (180kDa). Other published reports have shown (using different cell lines) that the protein can also be expressed as a 140kDa protein (Santoni et al 1989), and although recognised by the antibody used here, this protein species does not appear to be expressed in the cell lines examined. It can be seen in Figures 3.7.b-e. that the 180kDa NCAM species localises to the periphery of the cells, though not necessarily at areas of cell-cell contact.

Although the 16kDa protein, ductin, is believed to form the gap junction channel (section 1.3.1.1), antibodies against mammalian forms of the protein for immunofluoresence studies are not available. Examining expression of this protein at the transcriptional level via Northern analysis is complicated by the fact that it is ubiquitously expressed in mammalian cells, forming part of the vacuolar ATPase. Thus little information can be gained in how this protein is regulated in gap junctions using these types of analysis techniques.

Figure 3.5. Western blotting and imuunofluorescence analysis of Cx43 expression.

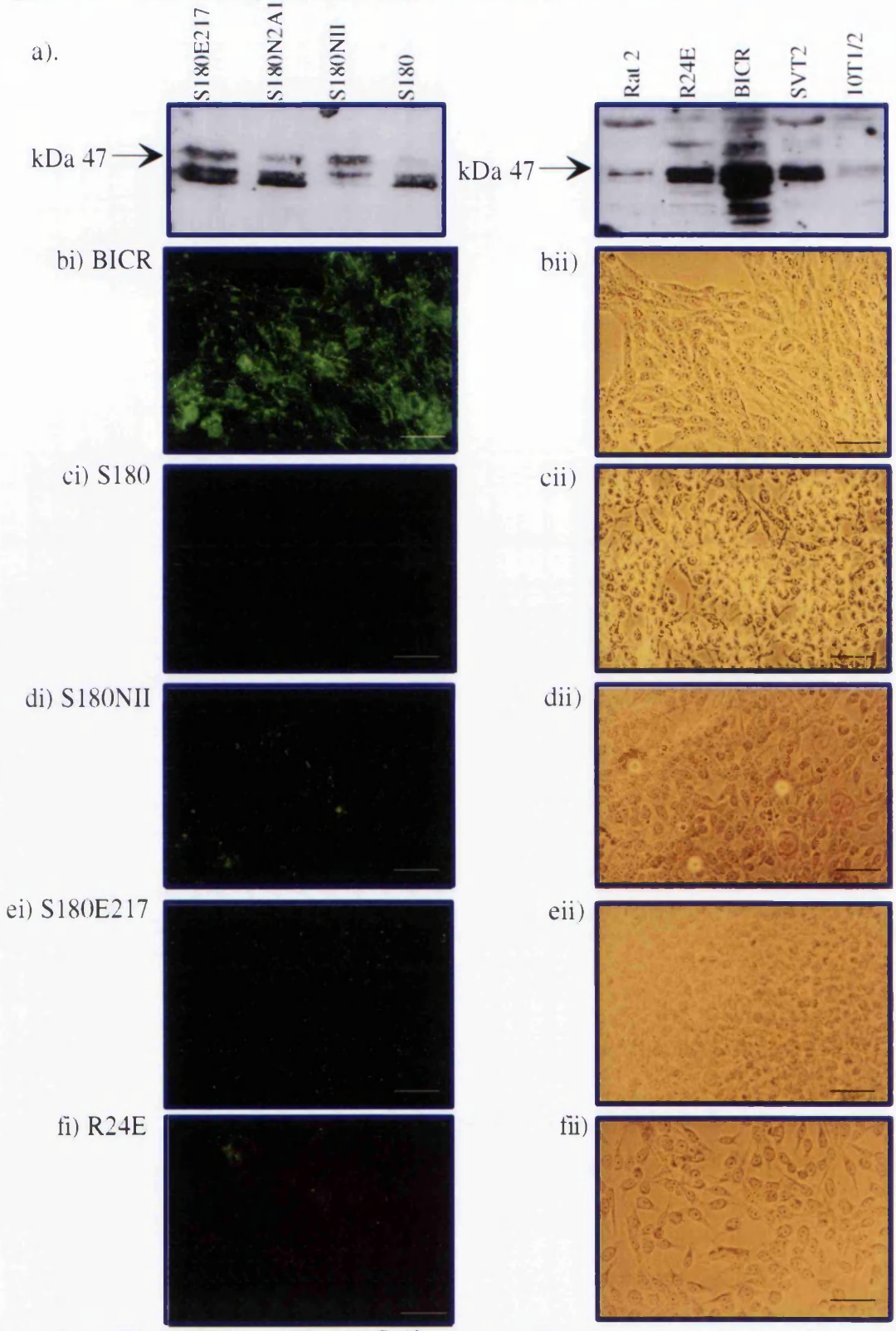


Figure 3.5. a) Western blotting analysis of Cx43 expression. Protein samples were prepared from each cell line (as described in section 2.8) and ~20µg (estimated from Coomassie blue stained gels) loaded into separate wells of a 10% PAG. Samples were not boiled prior to loading. Primary antibody was Zymega mouse monoclonal anti-Cx43 at 1:1000. Arrow indicates Cx43 (47kDa species). **Figures b-f** Cx43 immunofluorescence analysis. bi-fi are fluorescence micrographs of the phase contrast images shown in bii-fii. Cultures were prepared as described in section 2.8.4. Primary antibody was Zymega mouse monoclonal anti-Cx43 at 1:100. Bar 50µm.

Figure 3.6. Western and immunofluorescence analysis of Ecadherin expression

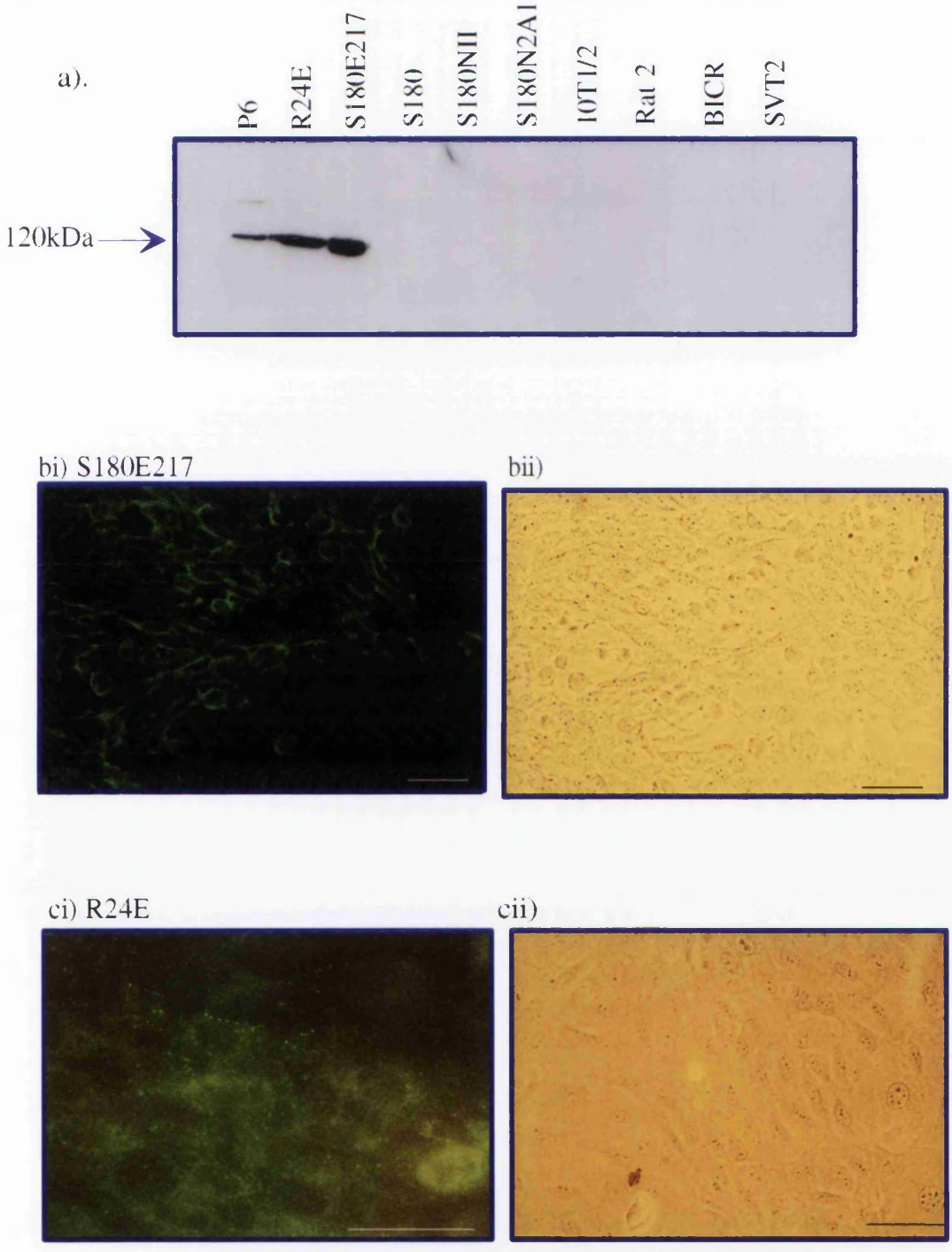


Figure 3.6. a) Western blotting analysis of E cadherin expression. Protein samples were prepared from each cell line (as described in section 2.8) and ~20 µg (estimated from Coomassie blue stained gels) loaded into separate wells of an 8% PAG. Samples were not boiled prior to loading. Primary antibody was Takara mouse monoclonal anti-E cadherin ECCD-2 at 1:5000. Arrow indicates E cadherin (120kDa species). **Figures b-c** E cadherin immunofluorescence analysis. bi-ci are fluorescence micrographs of the phase contrast images shown in bii-cii. Cultures were prepared as described in section 2.8.4. Primary antibody was Takara mouse monoclonal anti-E cadherin ECCD-2 at 1:100. Figure ci) was taken at a higher magnification than the coressponding phase image in Figure cii for which there was no equivalent objective available). Bar 50 µm.

Figure 3.7 Western and immunofuorescence analysis of N-CAM expression.

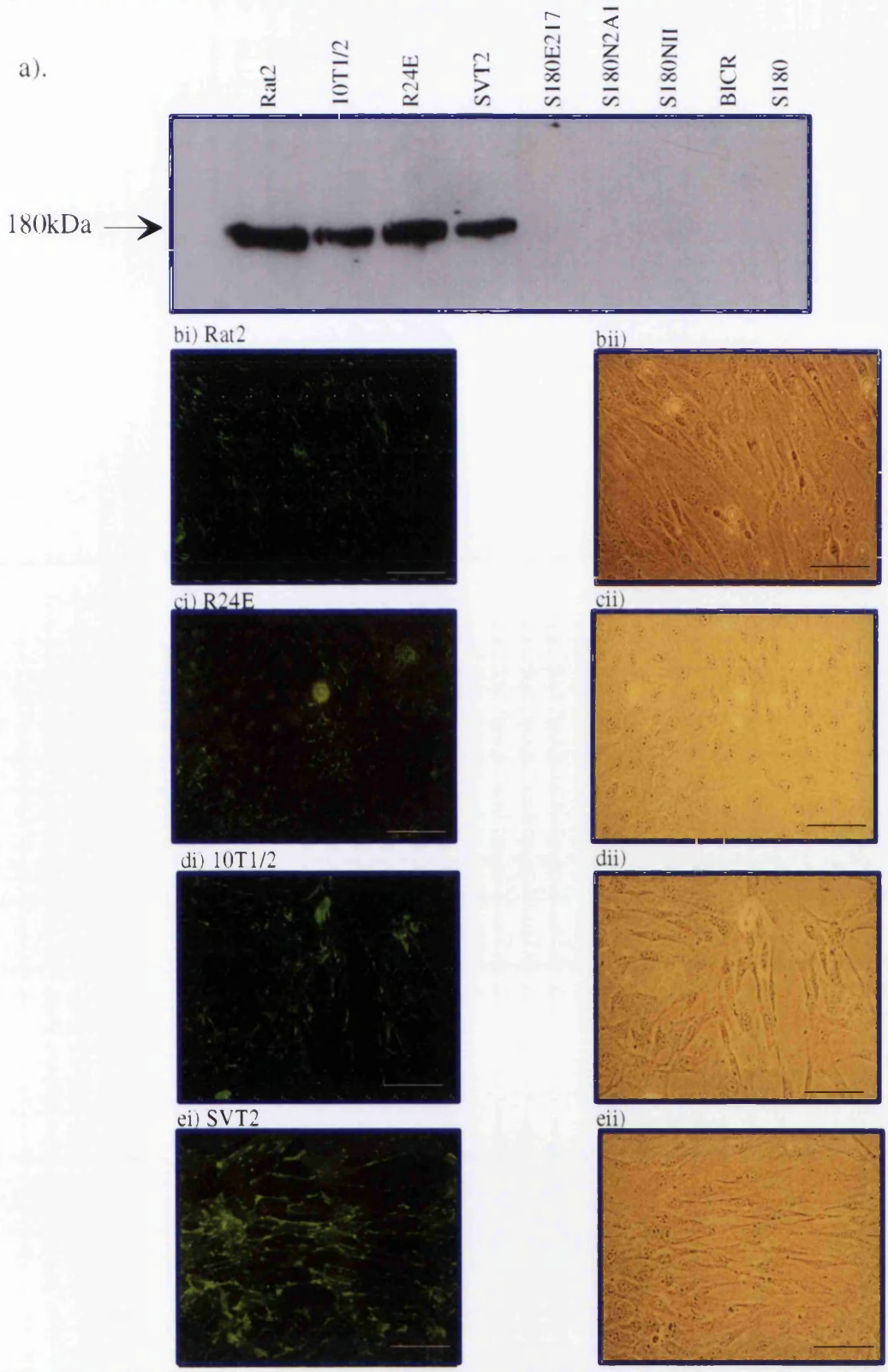


Figure 3.7. a) Western blotting analysis of NCAM expression. Protein samples were prepared from each cell line (as described in section 2.8) and ~20µg (estimated from Coomassie blue stained gels) loaded into separate wells of an 8% PAG. Primary antibody was Sigma mouse monoclonal anti-NCAM at 1:500. Arrow indicates NCAM (180kDa species). **Figures b-e** NCAM immunofluorescence analysis. bi-ei are fluorescence micrographs of the phase contrast images shown in bii-eii. Cultures were prepared as described in section 2.8.4. Primary antibody was Sigma mouse monoclonal anti-NCAM at 1:500. Bar 50µm.

Table 3.4. Summary of endogenous and exogenous expression of gap junction associated proteins.

	Rat2		R24E		10T1/2		S180		S180E217		S180N2A1		S180NII		SVT2		BICR	
	W	IF	W	IF	W	IF	W	IF	W	IF	W	IF	W	IF	W	IF	W	IF
Cx43	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
E cad'	-	-	+	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-
N cad'	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	+
NCAM	+	+	+	+	+	+	-	-	-	-	-	-	-	-	+	+	-	-

W - Western analysis, IF - immunofluorescence analysis. (+) indicates positive expression of the protein, (-) indicates no detectable levels of protein expression. Expression of N cadherin in S180NII and S180N2A1 cells was analysed by F. Tato, Rome. Expression of N cadherin in BICR was analysed by D. Prowse. All cells tested negative for Cx26 & Cx32, and P cadherin.

3.2.4. Summary.

A panel of cell lines has been established which meet the requirements outlined in section 3.1. S180 cells, which are poorly coupled, show increased gap junctional communication after transfection with cDNA for E or N cadherin. Well coupled transformed cells are represented by BICR cells and SVT2 cells. The growth phenotypes of the normal cell lines selected are such that they provide suitable background monolayers on which to test the growth of the transformed cells and the role of GJIC in the inhibition pathway.

The results presented here do not support a general hypothesis (Loewenstein 1979, Kalimi et al 1992) that levels of homologous communication are indicative of a cells tumourigenicity. The transformed and tumourigenic cell lines BICR and SVT2 (Brummer 1987, Fernandez et al 1992) have similar levels of communication to those of the normal, non-tumourigenic cell lines. Although the sarcoma derived S180 cell line shows very low levels of communication, the findings show that an aberrant growth phenotype is not always associated with loss of homologous GJIC.

In several studies where heterologous communication has been correlated to the inhibition phenomenon (Mehta et al 1986, Mikalsen 1993) only transfer frequency (the proportion of cells which showed transfer to at least one surrounding cell) has been used to measure communication, however this gives only a partial indication of the extent of communication. Frequency of communication is dynamic, i.e. a frequency of 50% does not necessarily mean that 50% of cells can communicate and 50% can't, rather, that at a point in time 50% are coupled. However, when cells are in contact, for however short a period, the level of communication (number of cells into which transfer occurs) is likely to depend on the number of channels and extent of coupling. The level of communication is likely to be more important for understanding the inhibition phenomenon. It has been suggested that the number of channels and extent of coupling is determined, at least in part, by the level of connexin expression.

3.2.4.1. *Relationship between Cx43, cell-cell adhesion and communication.*

Connexins are thought to be involved in the formation and/or regulation of vertebrate gap junctions (section 1.3). However, the mechanisms that regulate their function are poorly understood. The results obtained here using the S180 series of cell lines are consistent with reports published by Musil et al (1991). That is, expression of exogenous E cadherin in S180 cells results in increased conversion of Cx43-NP to the phosphorylated form (Cx43-P_2) and increased homologous communication. In addition, it has been shown here that N cadherin can also increase the conversion of Cx43 to Cx43-P_2 in S180 cells and increase their levels of homologous communication. This data would suggest that phosphorylated Cx43 is involved in gap junction formation and that the original defect in GJIC within the S180 cells was not due to a genetic defect in the Cx43

gene but rather a defect in the post-translational modification mechanism. The expression of Cx43-P_2 may modulate homologous communication by regulating the formation of the channels or their open and closed states. However, it is not known how E & N cadherin regulate the phosphorylation states of Cx43. The data from the S180 series of cell lines would suggest that Cx43 is required for gap junction formation and that cells which express phosphorylated species of the protein form gap junctions more efficiently. However, gap junctions also form with high efficiency in Rat2 and 10T1/2 cells despite the fact that Cx43 expression (phosphorylated and unphosphorylated) in these cell lines is relatively low. Furthermore, Rat2 cells which have been transfected with E cadherin show increased expression of Cx43 (Cx43-P_2 in particular), yet no increase in homologous communication.

BICR cells express high levels of Cx43 as indicated by Western blot analysis and are very well coupled. However, examination of Cx43 immunostaining reveals that the majority of the Cx43 localises at sites away from cell-cell contact points suggesting the most of the expressed protein is not involved in gap junction formation. The antibody used in the immunostaining study here is unable to determine whether the Cx43 that accumulates at cell-cell contact points is phosphorylated or unphosphorylated.

Although the Cx43 expression appears to be necessary for cells to communicate the data obtained would appear to show that other factors are involved in mediating high levels of gap junction formation. A likely candidate is the putative channel protein, ductin. However, expression analysis could not be performed due to the unavailability of appropriate antibodies.

3.2.4.2. *Gap junction specificity.*

The molecular basis of gap junction specificity is poorly understood. It has been suggested that the homotypic binding properties of adhesion molecules such as the cadherins and the ability of connexins to selectively bind to other connexins make them suitable candidates (section 1.4.5.2.b). However, data obtained from expression studies (section 3.4.3) would suggest that these proteins are not involved in the junctional specificity observed here. For example, low levels of heterologous communication are observed when the normal cells (Rat2, R24E and 10T1/2) are paired with S180E217 cells or SVT2 cells (Table 3.3). However, all of these cell lines express Cx43 (phosphorylated & unphosphorylated) and show high levels of homologous communication (Table 3.2).

The adhesion molecules NCAM and E cadherin are also unlikely to be responsible for the specificity observed. For example, S180E217 cells and R24E both express E cadherin and yet show very poor heterologous communication. The possibility exists that the E cadherin expressed in the R24E cells may not be fully functional due to the possible absence of cadherin associated proteins, e.g. the catenins, which are responsible for regulating cadherin function (Hirano et al 1992, Shimoyama et al 1992). The immunostaining in the R24E cells was weak in comparison to that in S180E217 cells. E-

cadherin would appear to be functioning in the S180 cells because of their altered morphology and increased homologous communication.

SVT2 cells and the normal cells express NCAM but show relatively poor heterologous communication. This would suggest that other factors, possibly other connexins (but not Cx26 & Cx32), are involved in specificity and that the process of gap junction formation is not as simple as the literature suggests.

The rate of gap junction formation is likely to be related to the frequency with which the apposed cell membranes come close enough together to allow interaction between channels that protrude 1nm. Increased adhesion through the expression of any adhesion molecule and perhaps by expression of any connexin, may increase this frequency and it may not be possible to associate specificity with any one given product.

The possibility exists that transformed cells reduce the levels of homologous communication of the normal cells. Such effects have been reported: Diener et al (1995) observed that malignantly transformed non-parenchyma cells suppressed the homologous communication of co-cultured rat liver parenchyma cells. However, these effects were seen over several days and it is not known by what mechanism this was achieved.

Despite being unable to determine the specific molecules responsible for the different levels of heterologous communication the importance of GJIC in the suppression of transformed cell growth can be assessed using these cell lines. Taken within the context of the current working hypothesis, one would predict the greatest inhibition to occur when R24E or Rat2 cells form the normal cell partner and the least inhibition when S180, S180E217 cells are co-cultured with 10T1/2 cells. Focus formation assays are now required to establish which transformed cells are suppressed by the presence of the normal cells and what the relationship is between gap junctional communication and growth suppression.

3.3. THE FOCUS FORMING ABILITY OF THE TRANSFORMED CELLS.

3.3.1. Introduction.

Focus assays were carried out on the cell combinations shown in Table 3.5 to establish whether the transformed cells have suppressible growth phenotypes when co-cultured with excess resting normal cells. And, if so, whether the inhibition correlates with the presence of heterologous GJIC.

In initial experiments foci were located after staining the co-cultures with Giemsa (a cell stain often used in this type of analysis), however, the colonies have to be of a sufficient size and density to avoid the possibility of interpreting clumps of normal cells or cell debris as small foci. To overcome any possible ambiguity an x-gal stain was used to locate those cells expressing the exogenous β-gal gene within the co-culture, i.e. the transformed cells. A preliminary focus assay was performed in order to determine if the different stains, when applied to replicate cultures, yielded significantly different results (based on focus number per plate & average focus size per plate). An example from this experiment can be seen in Figure 3.8. Although there was no statistically significant difference between the staining methods in terms of the number of foci recorded (P>5%) and the size of foci recorded (P>5%), small foci (0.125mm^2) were difficult to see by Giemsa staining alone. Foci stained with x-gal were much clearer and the use of the stain avoided the possibility that clumps of normal cells or cell debris could be misinterpreted as foci. Cultures were frequently counter-stained with Giemsa as it did not interfere with the x-gal stain.

In the focus assays, only foci above a diameter of 0.4mm (focus area of 0.125mm^2) were examined. In most of the assays some foci below 0.125mm^2 were observed, however, these were expected even if the growth of the transformed cells were inhibited since the normal cells continued to divide for 24-36 hours after seeding (section 3.1), allowing time for the transformed cells to pass through 2 or 3 divisions before any inhibitory effect by the normal cells could be imposed.

In focus formation assays normal and transformed cells were plated together at densities of 10^6 and 500 cells / per 90mm dish respectively. Duplicate (or triplicate when focus numbers were small) co-cultures and control cultures (separate cultures of normal and transformed cells seeded at the same initial densities as above) were set up in each experiment. After 12 days in culture the cells were fixed, and stained with x-gal for 8 hours (section 2.5.1) to locate the transformed cells. The numbers of foci (or colonies in control cultures) on each dish were counted and average focus area determined from 40 foci/colony samples, (or maximum possible when less than 40 formed).

The results of the focus formation assays are presented in Tables 3.6.a & 3.6.b. Photographs of representative examples are shown in Figures 3.9.a & 3.9.b.

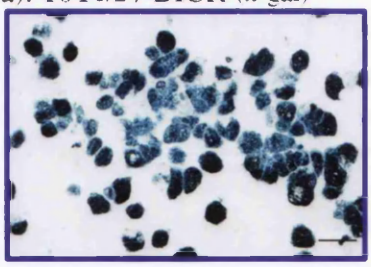
Table 3.5. Co-culture combinations for focus formation assays.

Transformed cells	Normal cells		
	Rat 2	R24E	10T1/2
S180	*	*	*
S180E217	*	*	*
S180NII	*	*	*
S180N2A1	*	*	*
SVT2	*		*
BICR	*		*

* Indicates focus assays carried out. Transformed cell lines are β-gal$^+$.

Figure 3.8. A comparison of two staining techniques, used to identify foci and colonies of transformed cells

a). 10T1/2 / BICR (x-gal)



b). 10T1/2 / BICR (giemsa)

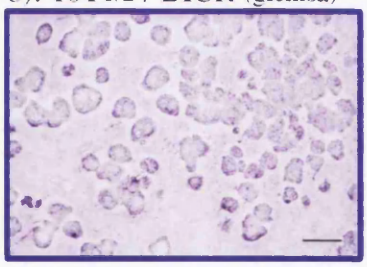


Figure 3.8. The figures above are examples from an experiment performed to compare two different cell staining methods. 10T1/2 cells and BICR-βgal^+ cells were seeded at 10^6 and 500 cells per dish respectively. All cultures were grown for 12 days. They were then fixed in 1% Formaldehyde and 0.1% Gluteraldehyde in PBS for 30 mins, washed with PBS and stained with either x-gal for 8hrs (Figure a), which stains blue those cells expressing an exogenous β-gal gene, or stained for 10 mins in a 1:10 solution of Giemsa (Figure b). See section 2.5.1 for staining procedures. Bar 3mm.

3.3.2. The effect of normal cells on focus number.

The S180 E & N cadherin transfectants form more colonies than S180 parental cells (Table 3.6.a. - controls) which would suggest that these cells can form better attachments to the culture dish or those that do attach grow better under low density conditions. It can be seen in the photographs of Figure 3.9.a. that there is a subtle difference in colony morphology between the S180-cadherin transfectants and the S180 parental cells. The S180E217 and S180NII cell colonies appear more compact, particularly at the colony periphery (a phenomenon most likely attributed to increased cell to cell adhesion in the transfectants). BICR cells have the highest colony forming ability whereas SVT2 cells have a relatively poor colony forming ability (Table 3.6.a; Figure 3.9.b).

The data in Table 3.6.a. shows that the number of S180 foci which form in the presence of the normal cells is similar to or slightly greater than the number of colonies formed in control cultures. In contrast, the S180 E and N cadherin transfectants show marked inhibition of focus number when co-cultured with Rat2 and R24E cells (10T1/2 cells do not inhibit the number of foci formed by these cells). Examples of foci formed by the S180 series of cell lines is illustrated in the photographs in Figure 3.9.a.

The ability of the Rat2 and R24E cells to inhibit the number of foci formed by the S180 cadherin transfectants is consistent with the hypothesis that heterologous communication between the two cell types is required. For example, S180 cells show low levels of communication with, and are not suppressed by, the normal cells (Table 3.3 & 3.6.a.), whereas S180NII cells are well coupled to the Rat2 and R24E cells and their focus forming ability is markedly inhibited. A correlation analysis was performed in which the relationship between the number of foci formed by the S180 series of cell lines and their level of communication with the normal cells was examined. A strong positive correlation was revealed (Table 3.7). However, some of the transformed cells (S180E217 & S180NII) also communicate with 10T1/2 cells (albeit relatively poorly; Table 3.3) but show very low and often no inhibition of focus number when co-cultured with them. This would suggest that heterologous communication, although necessary is not sufficient to inhibit the number of foci formed.

SVT2 cells, which form functional heterologous gap junctions with the normal cells, are not restricted in terms of the number of foci which they form, indeed focus formation is increased. Despite the high levels of heterologous communication recorded between BICR cells and the normal cells the inhibition of focus number is relatively poor (Table 3.6.a). Together these results would suggest that SVT2 cells have lost the ability to be inhibited by normal cells whereas the BICR cells would appear to respond poorly to the inhibition mechanism. These cell lines serve as useful controls, (in conjunction with the conditioned media data - section 3.3.4) for the possibility that inhibition is caused from nutrient deprivation. The increased efficiency with which SVT2 and S180

cells form foci in the presence of the normal cells would suggest some form of feeder effect is occurring. This effect may be mediated by factors secreted from the normal cells into the media. To test this possibility conditioned media experiments were performed with these cell lines. Conditioned media experiments were also performed to confirm that the inhibition observed was not due to factors secreted into the media by normal cells - the results are presented in section 3.3.4.

3.3.3. The effect of normal cells on focus size.

Examination of focus size (Table 3.6.b) reveals a more complex picture than the focus number analysis and suggests that all of the transformed cells are growth inhibited to varying degrees. The S180 foci, formed in the presence of any of the normal cells, are considerably smaller than control colonies. The lowest level of focus size suppression for the S180 series, is recorded when the S180 cells are co-cultured with the 10T1/2 cells. Foci formed by S180E217 or S180NII cells are also smaller than their respective control colonies and smaller than the foci formed by S180 parental cells. Although the inhibition of focus size occurs throughout the S180 set of cell lines, a correlation exists between focus size inhibition and heterologous communication ($r = 0.6$, $P < 0.1\%$; Table 3.7). The differences between focus size and control colony size is illustrated clearly by the representative photographs in Figure 3.9.a & 3.9.b.

In the previous section it was shown that BICR and SVT2 cells show little or no inhibition of focus number, respectively. However, these cell lines show considerable focus size suppression, but, there does not appear to be a relationship between focus size inhibition and heterologous communication for these cell lines. BICR and SVT2 cells show higher levels of communication with Rat2 cells than with 10T1/2 cells and greater focus size inhibition when cultured with Rat2 cells. However, Rat2 cells are coupled better to BICR cells than to SVT2 cells (Table 3.3) but impose greater focus size inhibition on the SVT2 cells (Table 3.6.b). This data would appear to show that suppression is more complicated than is often suggested.

The photographs in Figure 3.9.a & 3.9.b. show examples of the foci formed by the transformed cells. It can be seen that the foci are not only smaller than the control colonies but have a different morphology. In addition, they appear more compact, (indicated by darker staining); possibly as a result of the physical presence of the normal cells. If the normal cells are imposing a restriction on focus expansion then the value of focus size as an index of growth inhibition may be limited.

Some smaller-than-average colonies were observed in the all of the cell combinations examined. These colonies appeared to be too small to be the result of increased cell compression alone and they therefore raise the possibility that cells within these foci are growth suppressed. An examination of the cell number and/or proliferative status of the cells within the foci is required to determine whether this is so. Many

S180E217 and S180NII foci below the 0.4mm threshold were also observed (Figure 3.9.a) and further analysis is required to determine if these foci have grown over the 12 day co-culture or represent colonies of cells established before any inhibitory effect could be imposed by the normal cells.

Table 3.6.a. The effect of normal cells on focus number.

Transformed cell line	Control	Rat2	% of ctl	R24E	% of ctl	10T1/2	% of ctl
S180	152 (18)	155 (16)	102	198 (18)	130	217 (25)	142
S180E217	164 (8)	60 (4)	37	103 (10)	63	215 (28)	131
S180NII	173 (8)	4 (1.4)	2	3 (4)	2	175 (14)	101
S180N2A1	196 (14)	24 (19)	12	28 (4)	14	195 (14)	99
SVT2	75 (33)	111 (23)	148			99 (2)	132
BICR	291 (15)	250 (50)	86			264 (52)	91

Table 3.6.b. The effect of normal cells on focus size.

Transformed cell line	Control	Rat2	% ctl	R24E	% ctl	10T1/2	% ctl
S180	16 (2.8)	4 (1.5)	24	4.5 (1.3)	28	6.2 (2.3)	39
S180E217	10 (3.4)	0.5 (0.3)	5	0.6 (0.2)	6	3 (1.2)	31
S180NII	17 (1.5)	0.3 (0.1)	2	0.1 (0.1)	1	3 (1.0)	19
S180N2A1	15 (4.6)	0.2 (0.1)	1	0.2 (0.1)	1	2 (0.7)	14
SVT2	13 (3.9)	0.8 (0.4)	6			3.8 (0.6)	29
BICR	6 (1.6)	1.5 (0.9)	25			3.8 (1.2)	62

Table 3.6.a & b. For controls, replicate cultures of each transformed cell line were plated at 500 cells / 90mm dish. In co-cultures, transformed and normal cells were plated simultaneously, in replicate, at initial densities of 500 and 10^6 cells / 90mm dish respectively. After 12 days the control and co-cultures were fixed in 1% Formaldehyde and 0.1% Gluteraldehyde in PBS for 30 mins. Foci were located after staining with x-gal for 8 hrs (see section 2.5.1) and Giemsa (1:10 solution) for 10 mins. The number of foci and colonies, per plate, above 0.4mm diameter (focus area 0.125mm^2) were counted (Table 3.6.a) and 40 colony / focus diameters (or max. possible when less than 40 formed) were measured from which average area (mm^2) was calculated (Table 3.6.b). Standard deviations in parentheses. Transformed cell lines have been placed in order of increasing heterologous communication (see Table 3.3 for values).

Table 3.7. Correlation analysis: Level of communication *vs* inhibition of focus size and focus number.

	Correlation analysis. Heterologous communication *vs*	
Coefficients	% inhibition focus No.	% inhibition focus size
r	0.8	0.6
P	<0.1%	<0.1%

Table 3.7. A correlation analysis was performed on the focus formation data obtained from experiments with the S180 series of cell lines, examining the relationship between their level of communication with, and inhibition by, the normal cells. The individual growth-inhibition values (derived from Table 3.6.a. & 3.6.b.) were categorised by communication level and subjected to linear regression analysis. The correlation coefficient (r) and confidence level (P) are given for each analysis. The type of calculation performed is described in Wardlaw (1992).

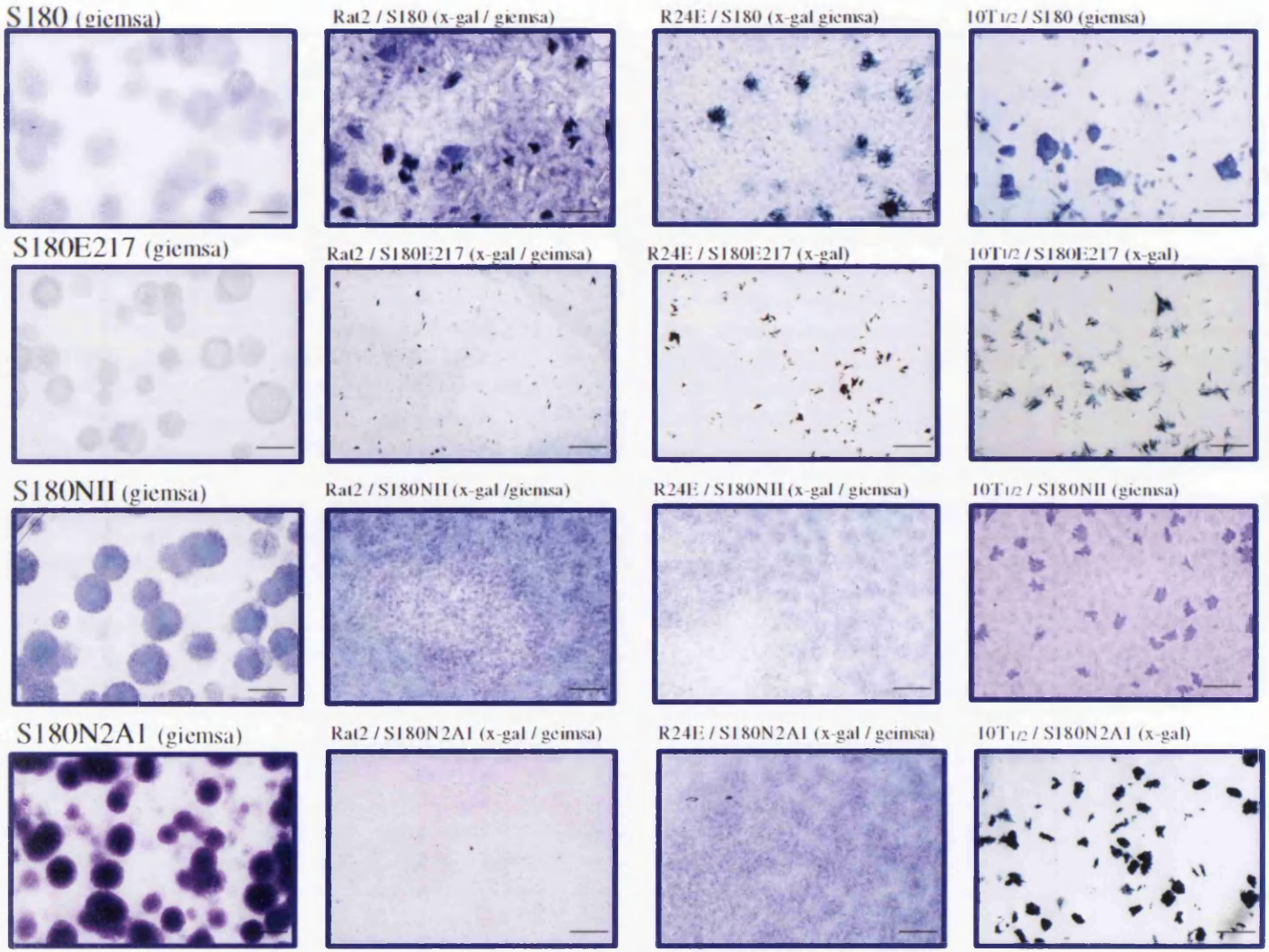


Figure 3.9.a. Representitive photographs showing the focus forming ability of the transformed cells on a background of either Rat2, R24E or 10T1/2 cells.

Transformed cells in control cultures were plated at 500 cells / 90mm dish. In co-cultures normal and transformed cells were plated simultaneously at 10^6 & 500 cells / 90mm dish respectively. The cultures were grown for 12 days and then fixed in1% Formaldehyde and 0.1% Gluteraldehyde in PBS for 30 mins. The cultures were then washed once in PBS and stained in either x-gal for 8hrs followed by counter-staining in Giemsa (1:10 soloution) for 10 mins, or stained only with Giemsa or x-gal. The type of stain applied to each plate shown is given in parentheses. See section 2.5.1 for staining procedure. Bar 5mm.

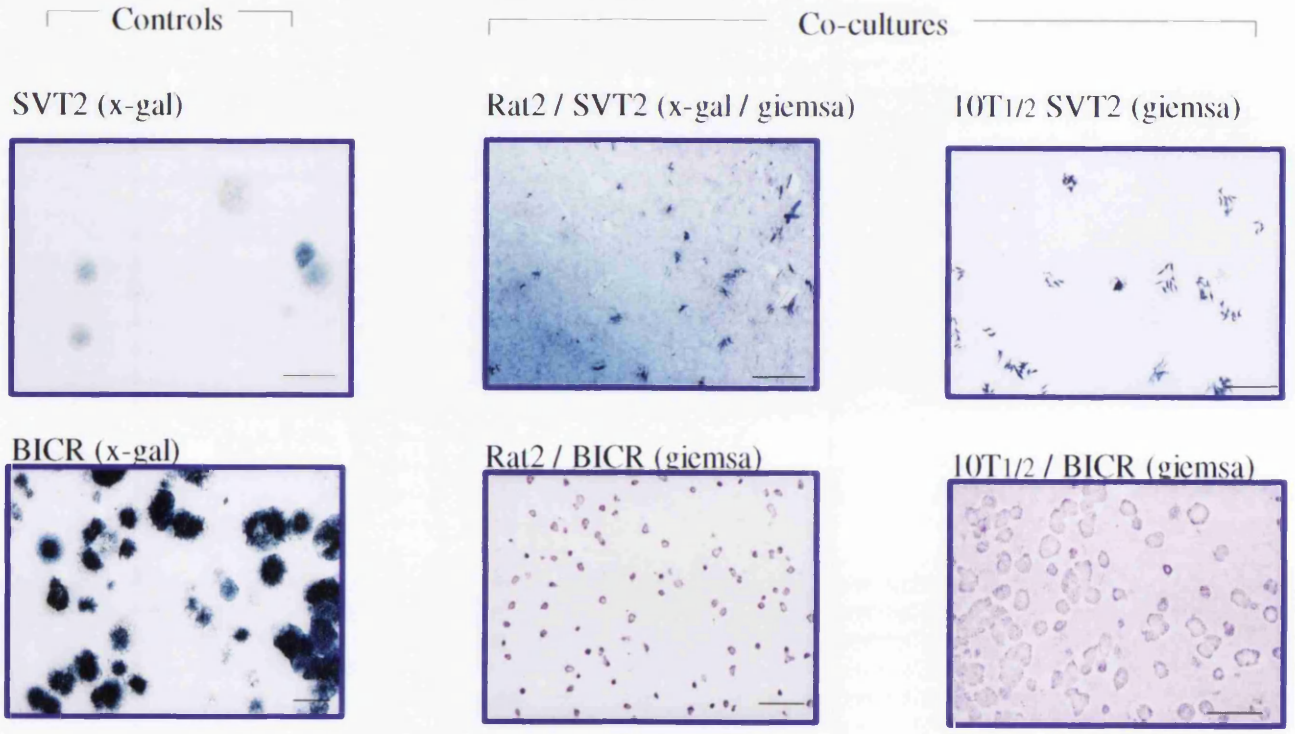


Figure 3.9.b. Representative photographs showing the focus forming ability of the transformed cells on a background of Rat2 or 10T1/2 cells.

See Figure 3.9.a for experimental details. The cell lines and cell combinations are given above each figure together with the types of stain used. Bar 5mm.

3.3.4. The effect of conditioned media on normal and transformed cell growth.

Experiments were performed in an attempt to answer the following questions:

1). Is focus formation inhibition mediated by factors secreted into the media by the normal cells?
2). Is the inhibition of focus number and focus size due to nutrient starvation?
3). Is transformed-cell conditioned media capable of stimulating the growth of high density Rat2 cultures?
4). Is increased focus formation mediated by factors secreted into the media by normal cells?

Proliferation assays, based on measurement of cell density and [^{3}H]-thymidine incorporation and analysis by autoradiography, were used to examine the effects of conditioned media on cell growth and focus formation. Four separate experiments were set up, each in duplicate, to answer the above questions. As a control for experiment B, cultures of 500 S180NII cells / 90 mm dish were grown in DMEM10% media and their proliferative status analysed after 1 and 4 days (experiment A; Table 3.8). To determine whether Rat2-cell conditioned media (conditioned for 10 days - see legend for Table 3.8) had any inhibitory growth effect on transformed cells, 500 S180NII cells/90mm dish (which showed the greatest levels of focus number and focus size inhibition in the previous section) were cultured in conditioned media and their growth analysed after 1 and 4 days (experiment B; Table 3.8).

As an control for experiment D, cultures of 500 Rat2 cells were grown in DMEM10% and their growth analysed after 1 and 4 days (experiment C; Table 3.8). The ability of the Rat2-cell conditioned media to sustain the growth of Rat2 cells (plated at 500 per 90mm dish) was examined (experiment D; Table 3.8); growth of these cells would indicate that their growth inhibition at high density is due to contact inhibition, rather than nutrient deprivation and that media taken from confluent 10 day old cultures (see Table 3.8) is still capable of sustaining normal cell growth. In experiment E (Table 3.8), the ability of S180NII-cell conditioned media, to stimulate the growth of Rat2 cells (500 cells / 90mm dish) was examined. All cultures were pulsed for 18hrs, prior to analysis, with [^{3}H]-thymidine. Cultures were then fixed and processed for autoradiography according to the method in section 2.5.

Finally, in an attempt to answer question 4, SVT2 cells were plated at 500 cells / 90mm dish and cultured in 10mls of Rat2 conditioned media for 12 days. They were then fixed, stained in x-gal for 8 hours and the number of foci counted.

Table 3.8. The effect of normal- and transformed-cell conditioned media on cell growth.

Experiment	Conditioned media	Cell line applied to [initial cell density /dish]	Hours in conditioned media 24		96	
			Cells/UA	LI %	Cells/UA	LI %
A	-	S180NII [500]	5 (2)	100	12 (4)	100
B	Rat2	S180NII [500]	2.8 (1)	100	15 (2)	100
C	-	Rat2 [500]	3.5 (2)	100	14 (8)	100
D	Rat2	Rat2 [500]	2.5 (1)	100	19 (7)	100
E	S180NII	Rat2 [10^6]	32 (7)	85	36 (11)	5

Table 3.8. Rat2 and S180NII conditioned media was prepared by growing separate cultures of each cell line to confluence in DMEM10%. Conditioned media was removed after 10 days and centrifuged at 3000xg for 10 min to remove debris and used directly or stored at -20°C (for a max. of 7 days). In each experiment, (set up in duplicate), cells were plated in DMEM10% and left to attach for 12 hours before any conditioned media was added. Cultures which received conditioned medium were first washed twice with PBS before conditioned media was added at 10mls/ 90mm dish. Cultures were then analysed after 24 and 96 hours. 18hrs prior to analysis cultures were pulsed with [^{3}H]-thymidine (0.185MBq/ml), washed twice in PBS and fixed in 0.1% Formaldehyde and 0.1% Gluteraldehyde in PBS for 30 mins. Cultures were then processed for autoradiography as described in section 2.5. Cell density (cells/UA) and LI was determined over the colonies (Exp A-D) and the monolayers (Exp E) by counting the total number of cells (labelled and unlabelled) in 10 Unit Areas (or maximum possible when colonies were small; 1 UA = 0.175mm^2). The experiments were as follows: A) Fresh DMEM10% on S180NII cells plated at 500 cells/ 90mm dish. B) Rat2 conditioned media on S180NII cells plated at 500 cells/ 90mm dish. C) Fresh DMEM10% on Rat2 cells plated at 500 cells / 90mm dish. D) Rat2 conditioned media on Rat2 cells plated at 500 cells/ 90 mm dish. E) S180NII conditioned media on Rat2 cells plated at 10^6 cells/ 90mm dish. (-) indicates where fresh DMEM10% was added. Standard deviations in parentheses.

The data in Table 3.8 (Exp. B) shows that Rat2 conditioned media has no detectable inhibitory effect on the high level of cell proliferation in low density cultures of S180NII cells (in comparison to controls - Exp A). Furthermore, the Rat2-cell conditioned media is able to sustain the growth of low density S180NII and Rat2 cultures (Exp. B & D), indicating that the inhibition of focus size and number, observed in the previous section, was unlikely to be due to nutrient starvation.

S180NII-cell conditioned media did not appear to stimulate the growth of high density Rat2 cultures (Exp. E). After 1 day in culture the LI of these Rat cultures is high (~85%), however, after 4 days the cells have reached their saturation density (~4.5^6; section 3.1.1) and their LI is appropriately low (~5%).

An experiment was also performed to determine if Rat2-cell conditioned media was capable of increasing the colony forming efficiency of SVT2 cells. The number of SVT2 colonies after 12 days in culture with Rat2 cell conditioned media was 87 (sd 23) which is not significantly different from the number of colonies recorded in control cultures for this cell line; 75 (sd 33); Table 3.6.a. This would suggest that the increased colony forming efficiency when co-cultured with Rat2 cells is dependent on the presence of the Rat2 cells and/or direct contact with them.

These experiments would suggest that Rat2 conditioned media is unable to inhibit cell growth and S180NII conditioned media is unable to stimulate cell growth. Furthermore, these experiments show that nutrient deprivation is unlikely to contribute to any inhibition of cell growth recorded.

3.3.5. The effect of focus size on the focus forming efficiency of the transformed cells.

In sections 3.3.2 & 3.3.3 many small foci below the 0.4mm focus diameter cut-off point were observed in x-gal stained cultures. It was not clear whether these small foci had grown over the 12 day culture period or represented colonies which had grown prior to any inhibition being imposed by the normal cells. In addition, many smaller than average foci were observed in cultures of cells which, on average, showed little focus inhibition. These results suggest that there is considerable variability within a population of transformed cells, in terms of the ability of individual cells to form foci in the presence of resting normal cells. To determine whether these small foci were inhibited or whether they were only partially suppressed and continued growing at a slower rate over the 12 day co-culture, focus assays were set up, and analysed over a 12 day time course. To increase the sensitivity of the analysis the threshold focus size, previously 0.4mm focus diameter (focus area = $0.125mm^2$), was lowered to 0.1mm focus diameter (focus area = $0.008mm^2$). This size is equivalent to ~100 cells i.e. the approximate size of a transformed colony after 4 days growth. This size accounts for transformed cell growth before the normal cells impose any inhibition and is therefore a logical cut off to choose.

All of the transformed cells examined showed some degree of focus size inhibition (section 3.3.3) which would suggest that the not all of the transformed cells totally escape inhibition during the first 4 days in co-culture. Within each co-culture there was a degree of variability in terms of focus size. This size heterogeneity may be caused by the degree to which foci are inhibited and at what stage in the time course they escaped. That is, inhibition may reach a maximum after 3 or 4 days. There may be a high frequency with which foci initially escape and a lower frequency of continuing escape. This would give a burst of larger foci (those which escape at day 3 / 4), some intermediate sized foci which escaped later and some small foci which did not escape at all. The level of heterologous communication between the normal and transformed cells may affect the frequency with which foci are inhibited. To determine whether a focus size exists, beyond which, the central cells at least escape inhibition and maintain aberrant growth phenotypes, colonies of these cells (and S180 parental cells) were pre-established for 2 and 4 days prior to the addition of Rat2 or 10T1/2 cells.

Focus assays were carried out on the cell combinations shown in Table 3.10.a. They include S180NII cells which appear to be well suppressed by Rat2 cells and S180E217 cells which show intermediate levels of focus size inhibition relative to the poorly suppressed S180 parental cells. To compare the effects of different normal cell lines 10T1/2 cells were co-cultured with S180 and S180E217 cells. These combinations also provide suitable communications phenotypes with which to assess the role of GJIC in the inhibition pathway.

Table 3.9 describes the nomenclature, which has been used here and throughout the remainder of the thesis, to indicate the number of days the transformed cells were grown in separate cultures and co-cultures. For example a time point of d6d4 would indicate that the transformed cells have spent a total of 6 days in culture (this is all the information that is required when examining control cultures) and 4 days in co-culture with the normal cells.

Focus assays were performed in which transformed cells were plated simultaneously with the normal cells or pre-established for 2 and 4 days prior to the addition of the normal cells. In co-cultures, transformed and normal cells were plated at 500 cells and 10^6 cells per 90mm dish respectively, control cultures were represented by separate cultures of each cell line, plated at the same initial density as above. All cultures were set up in duplicate. Recordings of focus number and size (area) were taken at 4, 8 and 12 days. This allowed any increase in colony or focus size over a 12 day period to be detected and measured. After the specific culture period co-cultures were fixed and stained with x-gal (section 2.5.1).

At time points where the two cells types were plated simultaneously, (d4d4, d8d8 & d12d12) both focus size and number have been used as an index of suppression, (Tables 3.10.a & 3.10.b). However, in time points, where transformed cell colonies were pre-established, the colony and focus forming efficiencies (foci above 0.008mm^2) of the

Table 3.9. Time points and nomenclature used in the focus formation assays.

Co-culture description	Total time transformed cells have spent in culture (& control times) d	Time transformed & normal cells have spent in co-culture d	Nomenclature Code
Transformed & normal cells plated simultaneously	4 8 12	4 8 12	d4d4 d8d8 d12d12
Transformed cells pre-established for 2 days	6 10 14	4 8 12	d6d4 d10d8 d14d12
Transformed cells pre-established for 4 days	8 12 16	4 8 12	d8d4 d12d8 d16d12

d = days in culture.

transformed cells showed no statistical difference (P>10%) and therefore only focus size was used to measure inhibition (Tables 3.11 and 3.12).

3.3.5.1. *The effect of normal cells on focus number after 4, 8 and 12 days when both cell types are plated simultaneously.*

The number of transformed cell colonies within all control cultures increases over the 12 day culture period (Table 3.10.a) i.e. some are grow slowly to begin with. After 12 days in culture the number of colonies, formed by all of the cell lines, above the 0.008mm^2 threshold area were similar to the number recorded in the previous section (3.3.2) using a higher threshold area of 0.125mm^2. This indicates that after 12 days in culture the majority of colonies that have grown are above the 0.125mm^2 minimum threshold area.

The number of foci in the co-cultures also increased over the 12 day culture (Table 3.10.a). However, more foci were recorded at day 12 when a threshold size of 0.008mm^2 was used (compare with data in Table 3.6.b when a threshold of 0.125mm^2 was used). This was particularly evident in cultures of Rat2/S180E217 cells and Rat2/S180NII cells. Although these cells form fewer foci the number of foci increases over the 12 day co-culture and suggests the cells grow more slowly in co-culture or their growth is initially delayed. These results are consistent with observations made in the previous section where many small foci (below 0.125mm^2) were observed.

3.3.5.2. *The effect of normal cells on focus size after 4, 8 and 12 days, when both cell types are plated simultaneously.*

To examine whether the small foci (particularly in the inhibited cultures) had grown over the 12 day culture period, their average size (area) was determined at 4, 8 and 12 days. The data is presented in Table 3.10.b. It is clear from the results that there is variability in terms of focus size within each experiment. Such variability is common in these types of experiments and may be due, for example, to differences in the time taken for individual cells to start dividing or differences in local normal cell density. Despite this variability, trends within each focus assay can be identified.

S180 foci above the threshold area were recorded after 4 days in culture with either Rat2 or 10T1/2 cells. Those S180 foci recorded at day 8 (in co-cultures with Rat2 cells) increased in size considerably over the following 4 days of culture (from an average of 0.1mm^2 at d8 to 4mm^2 at d12), however, they are significantly smaller at both time points, than the respective control colonies (16mm^2 at d8 & 18mm^{2}at d12).

Foci formed by S180E217 cells in co-culture with Rat2 cells continue to grow over the 12 day culture period (from 0.1mm^2 to 0.5mm^2) but remain markedly smaller than their respective control colonies which grow from 3.1mm^2 to 12.1mm^2 and foci formed by S180 parental cells. Thus, S180E217 foci show size suppression over a 12

day culture period when plated simultaneously with the Rat2 cells, however, the suppression isn't total and the foci do expand over time.

There were no S180NII foci above the minimum threshold area of 0.008mm^2 observed after 4 days in co-culture with Rat2 cells. After 8 days co-culture, foci with an average area of 0.1mm^2 were present and these continued to grow over the next 4 days to an average size of 0.2mm^2. These data would suggest that the small foci observed in the previous section are not simply colonies of transformed cells which were established prior to the normal cells imposing any growth inhibition. However, an examination of the proliferative status of the cells within the foci is required in order to confirm this interpretation and to determine the proportion of dividing cells.

In general the level of inhibition (focus number and size) imposed by the normal cells tends to decrease over the 12 day culture period. This would suggest that the larger a focus grows the less effective the inhibition mechanism, imposed by the normal cells, becomes.

The results presented in this section would suggest that the enhanced levels of heterologous communication between the S180 E & N cadherin transfectants and the normal cells (relative to S180-parental cells), leads to increased levels of focus size inhibition. However, this inhibition is not total and foci continue to expand, albeit at a reduced rate relative to control colonies.

3.3.5.3. *The effect of normal cells on focus size when the transformed cells are pre-established for 2 and 4 days.*

In the previous sections it has been shown that S180 cells transfected with E or N cadherin, show increased focus size suppression relative to the S180 parental cells. To determine whether these cells escape this inhibition upon reaching a critical colony size, colonies of transformed cells were pre-established for 2 and 4 days and co-cultured for 4, 8 and 12 days with normal cells. Focus size was used an index of suppression and the results of the analysis are presented in Tables 3.11 & Table 3.12. The photographs in Figures 3.10.a & 3.10.b provide representative examples of focus/colony size and morphology at different analysis time points.

Control colonies of all the transformed cell lines examined continued to grow over all of the time periods analysed (with the exception of BICR colonies between d12 and d16). It can be seen from the examples in Figure 3.10.b. that as colonies increased in size, cell density, particularly within the centre of the colonies also increased - as indicated by darker staining. In later time points (e.g. d16), cells within the centre of some colonies detached during fixation. It is not clear whether this was because cells were unhealthy or whether they were only weakly attached to the culture dish, due to over crowding.

Within the co-cultures the pre-established colonies of all the transformed cells examined continued to grow in the presence of the normal cells (Table 3.11.& 3.12).

Despite the level of variability in terms of focus size (% of control) within each of the separate focus assays it is possible to identify consistent trends within the experiment as a whole. There is still considerable focus size suppression in the majority of cultures examined. However, the results would appear to indicate that those cell lines which showed the greatest level of focus size suppression when plated simultaneously with the normal cells (i.e. S180E217 & S180NII cells; Table 3.10.b) have a reduced response to inhibition by normal cells when they are pre-established for 2 or 4 days (Table 3.11 & 3.12; Figure 3.10.a).

Over the majority of time points, however, S180NII cells are suppressed to a greater degree than both S180E217 cells and S180 parental cells. In several of the focus assays (e.g. Rat2 / S180E217 or 10T1/2 / S180E217; Table 3.12) the level of inhibition imposed by the normal cells decreases as the foci grow in size.

In general the ability of 10T1/2 cells to suppress the growth of pre-established S180 and S180217E cells is less than that of Rat2 cells. In some instances S180E217 cells (which are coupled to 10T1/2 cells) are inhibited less than the parental S180 cells, which show no detectable levels of heterologous communication with 10T1/2 cells. This suggests that GJIC does not play a dominant role in the inhibition observed here.

The results here would suggest that when inhibition occurs it is at its strongest when the two cell types are plated together simultaneously. Pre-establishing cell lines which, when plated simultaneously with normal cells are well inhibited, reduces their response to the inhibitory mechanism suggesting that the larger the focus of transformed cells the less inhibited the cells are.

In the majority of instances all of the transformed cells showed some size suppression even when pre-established. However, because cell lines which show low or zero levels of communication with the normal cells (and are therefore unlikely to be inhibited by a GJIC-mediated mechanism) also show considerable focus size suppression, the possibility exists that increased focus cell density, as a results of the physical presence of the normal cells, leads to differences in size between foci and colonies.

The detailed study carried out here highlights a major limitation of this type of experimental approach. That is, variability within a plate and between plates means that only general conclusions can be drawn. Foci within a plate vary in size and estimates of focus number depend on the arbitrary choice of a cut-off size, this can significantly alter the apparent result. Although focus size would appear to provide a more detailed picture of the differences in growth inhibition it is not clear if focus size directly reflects the level of growth inhibition. Because of the limitations of the focus assay it is not possible to state whether a focus ceases to be inhibited at a specific size. However, the data would appear to show that when a population of transformed cells (including those which have a suppressible phenotype when plated simultaneously with the normal cells) grows

beyond 100-200 cells a proportion of those cells become refractory to the inhibition mechanism.

Table 3.10.a. The effect of normal cells on focus number after 4, 8 and 12 days when both cell types are plated simultaneously.

Transformed cell lines	Analysis time points	Control		Rat2 co-culture			10T1/2 co-culture		
		avg	sd	avg	sd	% of ctl	avg	sd	% of ctl
S180	d4d4	100	15	96	10	96	100	14	100
	d8d8	113	11	131	8	116	95	5	84
	d12d12	159	4	156	13	98	175	15	110
S180E217	d4d4	85	7	72	5	85	100	11	118
	d8d8	163	3	139	20	85	208	8	128
	d12d12	170	0	129	14	92	232	22	166
S180NII	d4d4	60	14	-	-	-			
	d8d8	154	20	30	9	19			
	d12d12	180	22	27	4	15			
BICR	d4d4	130	6	140	10	108			
	d8d8	260	14	260	18	100			
	d12d12	300	0	360	25	120			

Table 3.10.b. The effect of normal cells on focus size after 4, 8 and 12 days when both cell types are plated simultaneously.

Transformed cell lines	Analysis time points	Control		Rat2 co-culture			10T1/2 co-culture		
		avg	sd	avg	sd	% of ctl	avg	sd	% of ctl
S180	d4d4	2.0	0.9	0.1	0.01	5	0.2	0.1	10
	d8d8	18.5	2.2	0.1	0.01	0.5	2.0	0.8	11
	d12d12	16.0	2.5	4.0	1.2	25	6.0	2.2	38
S180E217	d4d4	3.1	1.0	0.1	0.01	2	0.5	0.1	16
	d8d8	12.1	3.8	0.4	0.1	3	3.0	0.9	25
	d12d12	10.0	4.0	0.5	0.2	3	2.8	0.2	28
S180NII	d4d4	0.1	0.00	-	-	-			
	d8d8	8.7	2.0	0.1	0.01	1			
	d12d12	17.0	5.0	0.3	0.15	1			
BICR	d4d4	0.3	0.1	0.2	0.1	69			
	d8d8	4.4	2.2	0.9	0.2	21			
	d12d12	6.0	1.9	1.3	0.4	22			

Table 3.10.a & b. In co-cultures, transformed and normal cells were plated simultaneously at 500 and 10^6 cells / 90mm dish respectively. Control cultures were represented by separate cultures of each cell line plated at the same densities as above. Cultures were fixed in 1% Formaldehyde and 0.1% Gluteraldehyde in PBS for 30 mins after 4, 8 and 12 days. Foci were located after staining with x-gal for 8hrs (see section 2.5.1) and Giemsa (1:10 solution) for 10 mins. In Table 3.10.a. Total number of foci, above 0.1mm diameter (area 0.008mm^2), were counted. Average focus/colony area (Table 3.10.b) was obtained from 40 samples (or the maximum possible when less than 40 formed). Percentage of the control was calculated from the equation: $C_2 / C_1 \times 100$ where C_1= control colony area and C_2= focus area. Results are based on counts from at least two replicates. Cell lines have been placed in order of increasing heterologous communication (see Table 3.3 for values). (-) indicates where no colonies were detected.

Table 3.11. The effect of normal cells on focus size, when transformed cells are pre-established for 2 days.

Transformed cell lines	Analysis time points	Control		Rat2 co-culture			10T1/2 co-culture		
		avg	sd	avg	sd	% of ctl	avg	sd	% of ctl
S180	d6d4	2.5	0.8	0.4	0.2	17	0.1	0.03	5
	d10d8	10.9	1.5	2.0	1.0	18.4	4.2	1.0	38
	d14d12	15.4	1.5	3.5	1.5	22.5	3.5	1.0	22
S180E217	d6d4	2.0	0.8	0.01	0	0.4	1.1	0.1	57
	d10d8	3.6	1.2	3.1	1.8	88	4.9	0.3	138
	d14d12	10.1	3.5	4.9	3.1	48	7.1	1.0	70
S180NII	d6d4	1.7	1.0	0.7	0.2	42			
	d10d8	8.0	1.2	0.5	0.1	6.25			
	d14d12	16.9	2.8	2.2	0.13	13			
BICR	d6d4	1.3	0.1	0.2	0.1	15			
	d10d8	4.5	1.5	1.8	0.5	40			
	d14d12	5.0	1.2	1.1	0.8	22			

Table 3.11. The effect on focus size was determined when the transformed cells were pre-established for 2 days. Controls were represented by separate cultures of each transformed cell line plated at 500 cells / 90mm dish. In co-cultures, transformed cells were pre-established for 2 days (500 cells/dish), normal cells were added (in < 0.5 mls of media) at 10^6 cells/dish. Cultures were fixed in 1% Formaldehyde and 0.1% Gluteraldehyde in PBS for 30 mins after 4, 8 and 12 days. Foci were located after staining with x-gal for 8 hrs (section 2.5.1) and Giemsa (1:10 solution) for 10 mins. A hand-held magnifier with an eye-piece graticule was used to locate microscopic colonies. The smallest division on the graticule, 0.1mm, was used as the threshold diameter (area = 0.008mm^2), foci smaller than this were ignored. Average colony / focus area was obtained from 40 colonies / foci or the maximum possible when less than 40 formed. (sd) standard deviation, (avg) average. Percentage of the control was calculated from the equation: C_2 / C_1 x 100 where C_1= control colony area and C_2= focus area. Results are based on measurements from at least two replicate cultures. Cell lines have been placed in order of increasing heterologous communication (see Table 3.3 for values).

Table 3.12. The effect of normal cells on focus size, when transformed cells are pre-established for 4 days.

Transformed cell lines	Analysis time points	Control		Rat2 co-culture			10T1/2 co-culture		
		avg	sd	avg	sd	% of ctl	avg	sd	% of ctl
S180	d8d4	18.5	2.2	1.8	0.8	10	3.8	0.5	20
	d12d8	16.5	2.5	2.5	1.5	15	4.2	2.2	25
	d16d12	26.4	4.5	4.5	2.0	17	9.6	4.0	36
S180E217	d8d4	12.1	3.8	3.1	2.0	26	3.1	0.1	25
	d12d8	10.0	4.0	3.1	1.0	31	4.9	1.0	24
	d16d12	19.2	6.0	12.6	6.6	65	8.0	0.5	42
S180NII	d8d4	8.7	2.0	0.7	0.05	8			
	d12d8	17.0	5.0	0.85	0.08	5			
	d16d12	27.5	5.9	2.8	0.2	6.0			
BICR	d8d4	4.4	2.2	0.8	0.5	18			
	d12d8	6.0	1.9	1.3	0.8	22			
	d16d12	4.5	0.4	1.3	0.8	29			

Table 3.12. The effect on focus size was determined when the transformed cells were pre-established for 4 days. Controls were represented by separate cultures of each transformed cell line plated at 500 cells / 90mm dish. In co-cultures, transformed cells were pre-established for 4 days (500 cells/dish), normal cells were added (in < 0.5 mls of media) at 10^6 cells/dish. See legend for Table 3.11 for experimental details.

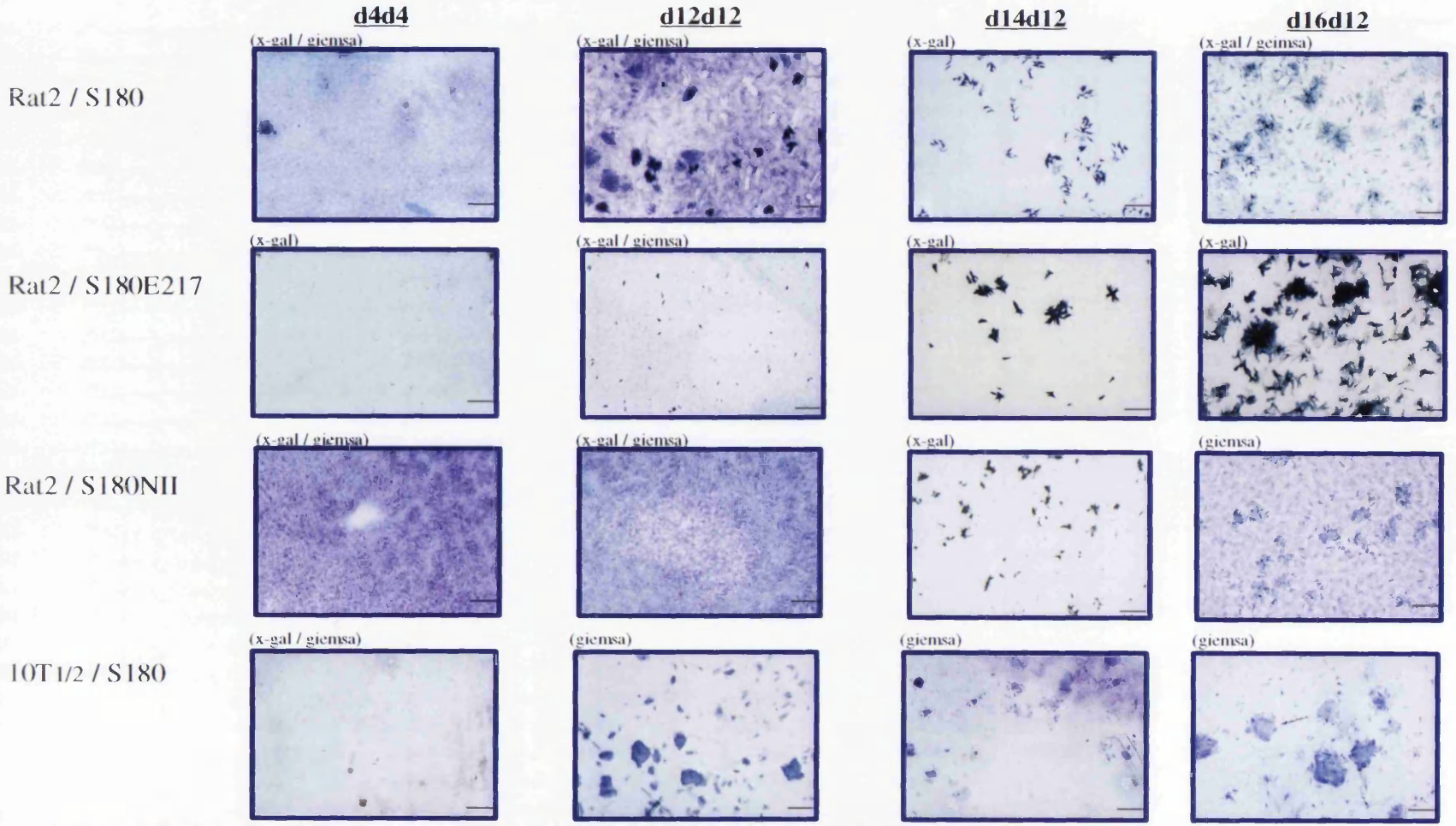


Figure 3.10.a. Focus forming ability of transformed cells when plated at the same time or before the normal cells.

All normal and transformed cells were plated at 10^5 and 500 cells /dish respectively. Controls were represented by separate cultures of each cell lines at the same densities as above (see Figure 3.10.b for respective control cultures). Cell combinations are given in the first column. Culture times are given at the top of each column. All cultures were fixed in 1% Formaldehyde and 0.1% Gluteraldehyde in PBS for 30 mins, washed once in PBS and stained with either x-gal for 8hrs and / or Giemsa stain (1:10 solution) for 10 mins (the type of stain applied to each plate shown is given in parentheses above each figure; see section 2.5.1 for staining procedure). Bar 5mm.

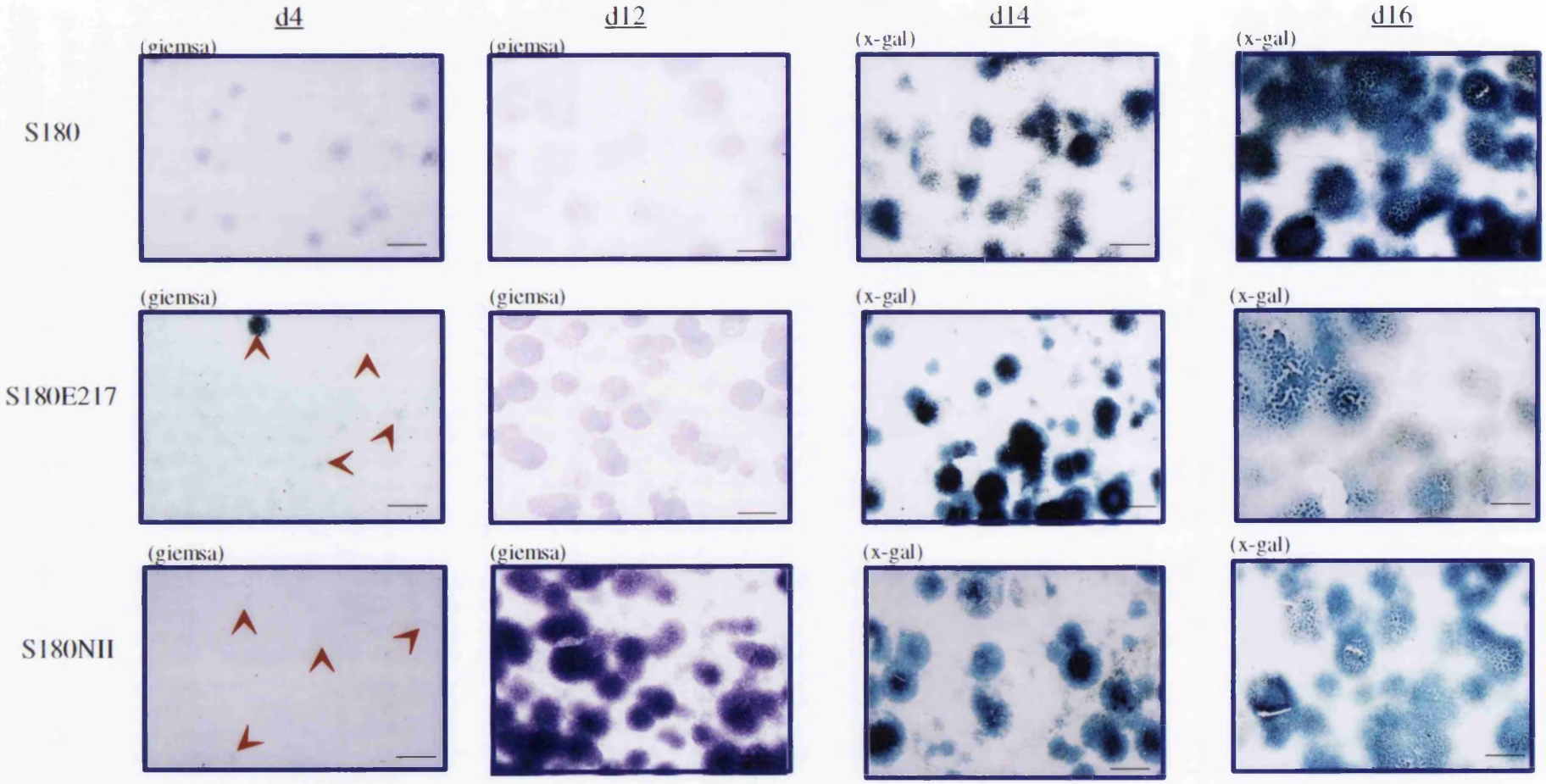


Figure 3.10.b. Colony forming ability of transformed cells - control cultures for Figure 3.10.a. See Figure 3.10.a for experimental details. Cell lines are given in the first column. Culture times are given at the top of each column. Cultures were stained with either X-gal for 8 hrs (blue stain) or Giemsa stain (1:10 solution) for 10 mins (The stain used in the plates shown are given in parentheses; see section 2.5.1 for staining procedure). Red arrows point to examples of colonies which are difficult to see at this resolution.. Bar 5mm.

3.3.6. Summary.

Preliminary focus assays (using a cut-off size of 0.4mm focus diameter), in which the S180 and S180 E & N cadherin transfectants were plated simultaneously with the normal cells (section 3.3.2) revealed a strong positive correlation between the inhibition of focus number and the level of heterologous communication. However, this correlation did not apply to the well coupled transformed cell lines, BICR and SVT2, which were poorly inhibited in terms of focus number. This would suggest that these tumourigenic cell lines (Fernandez et al 1990 & Brummer 1987) have lost the ability to respond to the inhibition mechanism.

The level of focus number inhibition varies depending on the arbitrary cut-off point which is used. Using a lower cut-off (focus diameter of 0.1mm) revealed that only S180 N cadherin transfectants were significantly inhibited in terms of focus number. However, analysis of focus size reveals that, in the majority of instances, all of the cell lines appear to be inhibited. Focus size inhibition is also seen in cell combinations which show low or undetectable levels of heterologous communication (e.g. 10T1/2 / S180) and in cell combinations where focus number is not suppressed (e.g. Rat2 / SVT2).

Conditioned media experiments would appear to show that the inhibition is not mediated by factors secreted into the media or due nutrient deprivation. This is consistent with the observations of Stoker (1966 & 1967) which suggest that the inhibition is mediated by direct cell-cell interaction between the two cell types. However, the type of conditioned media experiments carried out here do not rule out the possibility that the inhibition may be mediated by paracrine signals which act in a localised manner (due to rapid internalisation or degradation - see section 1.2.4).

A more extensive focus formation analysis, using a lower cut-off size, carried out in section 3.3.5 revealed that the cell lines which appeared to be well suppressed, in terms of focus number and/or size, during the initial focus assays (section 3.3.2; e.g. S180NII & S180E217 cells) appeared to be growing, albeit slowly, over the 12 day co-culture period. A greater number of foci were recorded and if the transformed cells were established for 2 or 4 days in the absence of normal cells, there subsequent response to the inhibitory mechanism was reduced. This would suggest that when a population of transformed cells (including those with suppressible phenotypes) grow to a sufficiently large size (>100cells) a proportion of those cells become refractory to the inhibition mechanism. If GJIC does mediate the inhibition then it might be expected that those cells towards the centre of the focus would escape, particularly if the transformed cells are poorly coupled to each other. As the focus grows then the transformed cells may stimulate each other to divide possibly by the production of high levels of stimulatory growth signals. This increase in local growth factor concentration may also lead to the stimulation of normal cells surrounding the foci.

The observation that larger transformed cell populations appear more likely to escape inhibition may be analogous to the observation *in vivo* in which initial masses of 10^3-10^6 transformed cells are required for tumourigenicity assays, any less and generally no tumours occur. Cells which don't go onto form tumours may simply die or be attacked by the immune system. Alternatively they may be suppressed by a gap junction mediated form of growth suppression. However, if a clump of cells is established within the host then their ability to be suppressed depends not only on heterologous communication but also on the transfer of the putative inhibitory signals or loss of putative stimulatory signals, throughout their population, (mediated via homologous communication). A similar pattern of inhibition may be occurring in tissue culture when colonies of transformed cells are allowed to grow prior to the addition of normal cells. If this were the case, in instances where transformed-cell homologous communication is low, one would expect to see more inhibition of cell growth at the periphery of colonies than in the centre. An investigation into patterns of growth inhibition across the foci has been carried out in the following results section.

Secreted factors do not appear to mediate the focus formation inhibition, and There is evidence to suggest that GJIC is involved in the inhibition of focus size where heterologous communication is high (e.g. S180E217 and S180NII cells), however, it is unlikely to be the inhibitory pathway in certain combinations which show focus size inhibition (e.g. 10T1/2 /S180). In these instances it is possible that the physical presence of the normal cells restricts focus expansion and the transformed cells continue to multilayer (rather than their growth being inhibited). Cell compression within the foci may therefore account for why focus size inhibition is observed in instances where there is no heterologous communication and where there is no focus number suppression (and often increased focus formation).

Although the focus assay experiments have shown that an inhibition phenomenon appears to exist within some of the cell lines examined, it can not be accurately measured using focus number or focus size. Until the growth of the individual cell populations are examined it is not possible to determine the role of GJIC in the phenomenon. The proliferative status of the transformed cells when cultured with the normal cell lines has been examined in the following section together with an investigation into the role of cell compression in focus size suppression.

3.4. CELL-CELL INTERACTIONS BETWEEN THE NORMAL CELL MONOLAYER AND TRANSFORMED FOCI.

3.4.1. Introduction.

In the previous section, discrete transformed cell populations above 0.1mm in diameter were regarded as foci and therefore likely to be formed by cells which had escaped the inhibitory growth control of surrounding normal cells. In several focus assays, such as those using S180, S180E217 and SVT2 cells, the number of foci which formed were similar to or greater than the number of control colonies formed. In addition, many such foci continued to increase in size over time suggesting cells were not growth suppressed. The majority of foci which formed, however, were markedly smaller than respective control colonies, which would suggest a high degree of growth inhibition. If focus size suppression is indicative of cell growth suppression, the current gap junction-mediated inhibition hypothesis would not appear to apply to all of the cell combinations examined here (e.g. 10T1/2 / S180). The possibility exists that focus size may not be an accurate reflection of cell growth. It was shown in section 3.3 that the majority of foci appeared more compact than control colonies, as indicated by darker staining. It is possible that increased focus cell density due to the presence of the normal cells may contribute to the explanation of focus size inhibition.

To examine this possibility cell density within the foci has been examined using the β-gal lineage marker to identify the transformed cells. A proliferation assay, based on [^{3}H]-thymidine incorporation and analysis by autoradiography, was used to determine the proportion and distribution of dividing transformed cells. The results of this analysis are presented in section 3.4.3.

In addition to the inhibition phenomenon, Stoker (1967) also observed that a small proportion of normal cells (when co-cultured with the transformed cells) were proliferating above background levels. It was suggested that this was possibly due to the growth media being changed prior to exposing the cells to [^{3}H]-thymidine. However, in Stoker's investigation, the normal cells that were used, maintained LI's of 10-20% even at high cell density, making it difficult to confidently identify small changes in cell growth. The normal cell lines used in this study, maintain very low labelling indices once they have reached their saturation density (section 3.1.1) and this should facilitate the analysis of any possible normal cell growth stimulation surrounding the foci.

To address the above points and the questions raised in the Introduction to the results (section 3.1), and to limit the number of experiments to a manageable size, only selected combinations of those used in section 3.3. have been analysed here. These include cell lines from the S180 series (S180, S180E217 & S180NII) in co-culture with Rat2 cells. In addition to these cell combinations, co-cultures of Rat2 / BICR cells and co-cultures of 10T1/2 / S180 cells will be used to assess the contribution, if any, of

increased cell density (relative to control colonies) contributes to the explanation of focus size suppression.

3.4.2. Does focus compaction contribute to the difference in size between foci and control colonies?

Focus cell density was measured to determine whether increased cell density (relative to the control colonies) could contribute to the explanation of focus size suppression. Focus assays were set up as described in section 3.3.5 using the cell combinations given above. For cell proliferation analysis, all cultures were pulsed with [^{3}H]-thymidine (0.185MBq/ml) for 18 hours prior to fixation, once fixed they were stained with x-gal and processed for autoradiography (section 2.5). For each time point 40 foci (or max. possible when less than 40 formed) and 40 control colonies, above a diameter of 0.1mm (area=0.008mm^2), were measured and average focus/colony area determined. To provide greater statistical confidence this data was combined with the data obtained from the focus formation experiments, presented in section 3.3.5. The combined averaged results have been presented graphically in Figure 3.11.

Cell density, within foci and control colonies was measured and is given as the number of cells / UA (1UA=0.175mm^2; see section 2.4). For each time point 5 foci and 5 colonies were analysed. Foci/colonies selected for analysis were not in contact with other foci/colonies and close to average size for each time point. For each focus/colony examined, cell density was derived from 10 UA's (or max. possible when focus/colony size was small) and the results from the 5 foci/colonies averaged and presented in the graphs of Figure 3.12; relevant points made throughout the text are illustrated in Figure 3.13. In some instances cell density together with the intensity of the β-gal staining within the focus was too high to distinguish individual cells, for example Figure 3.13.a. Attempts were made to overcome this problem by decreasing the staining time but x-gal staining is difficult to control and tends to be all or none. The intensity of staining varies between different cell lines which is presumably due to different levels of β-gal expression. For example, in Figure 3.13.b. a focus with high cell density is shown and although blue, individual cells can be distinguished. It was sometimes possible to overcome the problem by increasing the light intensity passing through the sample on the microscope stage.

It can be seen in Figures 3.11 that within each assay there is sometimes significant variability in control colony size. For example, S180 and S180E217 control colonies (Figures 3.11 A & B) at day 8 are larger than respective colonies recorded at day 12. This level of variability is perhaps to be expected in a colony formation assay, due to variation in the time taken for individual cells to attach and divide and colony distribution within the plate i.e. if two cells attach to the dish close to one another there may be local feeder effects. In focus assays the frequency of inhibition may vary and foci may

therefore grow at different rates. Despite the variability it is possible to determine significant trends within each experiment.

Cell density within S180 control colonies initially increases over time (Figure 3.12.A) but begins to level off after 10 days in culture. It was not possible to measure cell density in S180 foci at all time points. However, the fact that individual cells could not be identified in these foci (indicated by the asterisks in Figure 3.12.A) is itself indicative that cell density was high. These observations would suggest that high cell density, mediated by the physical presence of the surrounding Rat2 cells contributes, at least in part, to the difference in size between S180 foci and respective S180 colonies. It is not clear at this stage whether there is any cell growth inhibition within the foci. One method to determine the level of growth inhibition is to determine the total number of cells per focus (area x density) and compare it to the number of cells in respective control colonies. However, the calculation only provides a very rough approximation of total cell number, since the variations associated with focus area and cell density (which could not always be measured) are also multiplied. The proliferation assay enables a more accurate and detailed growth analysis to be carried out and, by examining the general degree of [^{3}H]-thymidine labelling above each focus, provides a better indication of cell growth within dense foci.

It can be seen from the data in Figure 3.12.B. that cell density within S180E217 foci, at all time points analysed, is higher than in respective control colonies. This difference in cell density can be seen in the focus and colony examples shown in Figures 3.13.c & d. It would appear that the high cell densities within the foci contribute to the explanation of focus size suppression (Figure 3.11.B). However, the difference in size between the foci and colonies at certain time points is so great (e.g. Figure 3.10.a) that it would suggest some degree of cell growth inhibition is imposed on these cells. The proliferative status of the S180E217 cells has been examined (section 3.4.3).

S180NII cells showed the highest level of focus number inhibition (section 3.3.2) and focus size inhibition (Figure 3.11.C). However, when plated simultaneously with the Rat2 cells they showed a very small increase in average focus size over the 12 day culture period and a marked increase in focus size over time when colonies were pre-established for 2 or 4 days (Figure 3.11.C). Cell density within the S180NII foci was generally less than in respective control colonies (Figure 3.12.C). Over the first 3 time points, when both cell types were plated simultaneously, cell density remains constant (~10 cells / UA). Cell densities within the pre-established foci was higher but also remained relatively constant over time (~20 cells / UA), and below respective colony cell density. Together, these data would suggest that increased cell density does contribute towards the explanation of focus size inhibition with this cell line.

Cell density within S180NII and S180E217 colonies increases over time but generally remains less than the level of cell density in S180 parental colonies. It would perhaps be expected that the cadherin transfectants would show higher colony cell

density than parental S180 cells on the basis that they adhere to each other better, due to the expression of N or E cadherin. However, these cells appear less inclined to multilayer, particularly at the colony periphery, thereby leading to a lower cell density when compared to that in S180 foci (e.g. Figure 3.13. g & h).

BICR cells show very little inhibition of focus number when cultured with the Rat2 cells, however they did show significant suppression in terms of focus size (Figure 3.11.D). Cell density within the foci (Figure 3.12.D) is higher than in control colonies which would suggest that cell compression and increased cell density contributes to the explanation of focus size inhibition (Figure 3.11.D). Increased cell density within the foci can be seen by comparing the focus shown in Figure 13.e with the respective control colony shown in Figure 13.f.

10T1/2 cells do not inhibit the number of foci formed by S180 cells (section 3.2), which would suggest that S180 cells escape growth inhibition. However, those foci which formed were significantly smaller than their respective control colonies (Figure 3.11.E). High cell density within the foci (Figure 3.12.E) indicates that the foci are compressed by the surrounding normal cells and this would appear to contribute significantly to the difference in size between S180 foci and S180 colonies. However, smaller than average foci were observed which would indicate the cells can be growth inhibited. The level of cell proliferation within these small foci and the averaged size foci has been examined in the following section.

The majority of foci examined were smaller than respective control colonies. In many published reports (e.g. Mehta et al 1986, Martin et al 1992) this type of result is frequently interpreted as suppression. However, further analysis of the foci shows that in many instances (with the exception of S180NII foci) cell density within the foci is markedly higher than cell density in control colonies. The data would suggest that the normal cells restrict the transformed cells from gaining access to the culture dish causing them to multilayer and their density within the foci to increase. The level of focus compression observed would appear to contribute to the explanation of focus size suppression and would imply that cell growth inhibition is not as high as the focus size data would suggest. To determine the level of growth inhibition within the compressed foci, a detailed analysis of cell growth within the foci and control colonies has been carried out. The results are presented next.

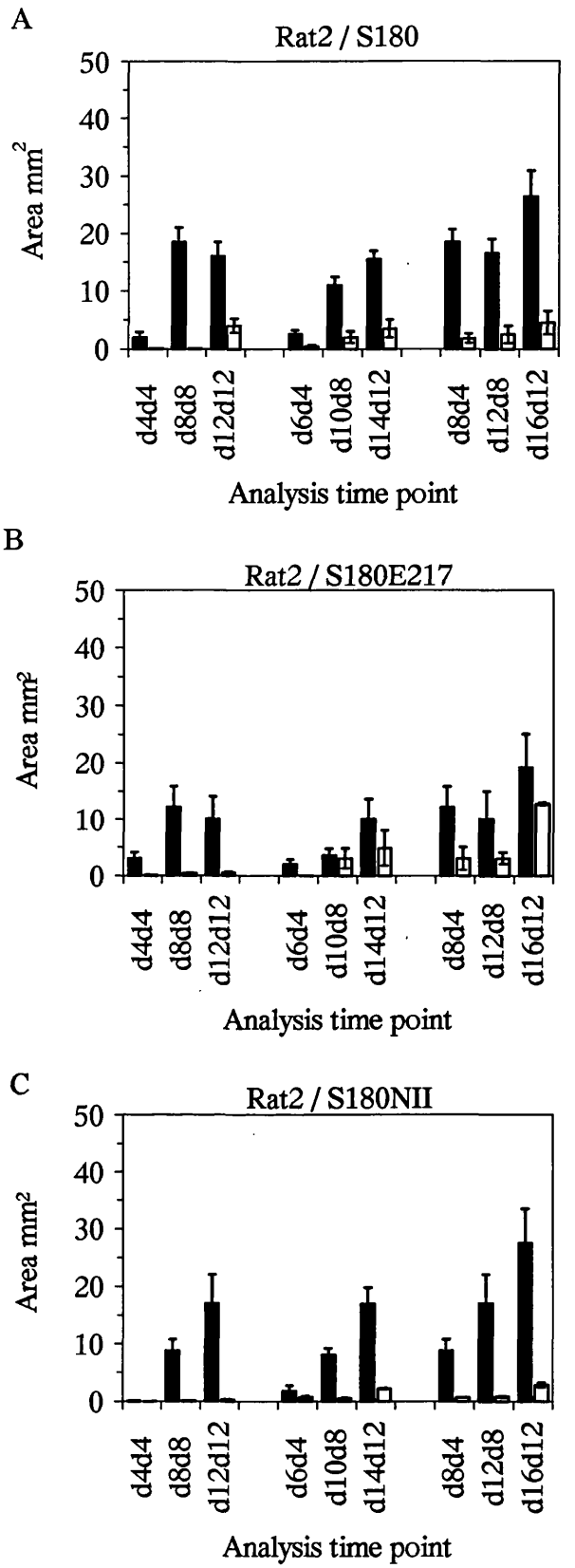


Figure 3.11. Average colony and focus size.

In co-cultures, normal and transformed cells were plated at 10^6 and 500 cells / 90mm dish. Separate cultures of each cell lines plated at the same density as above were set up as controls. The average colony and focus area at each time point was obtained from measuring 40 colonies and 40 foci (or max. possible when less than 40 formed) above a diameter of 0.1mm.The same S180 colony control data has been presented in Figures A & E.

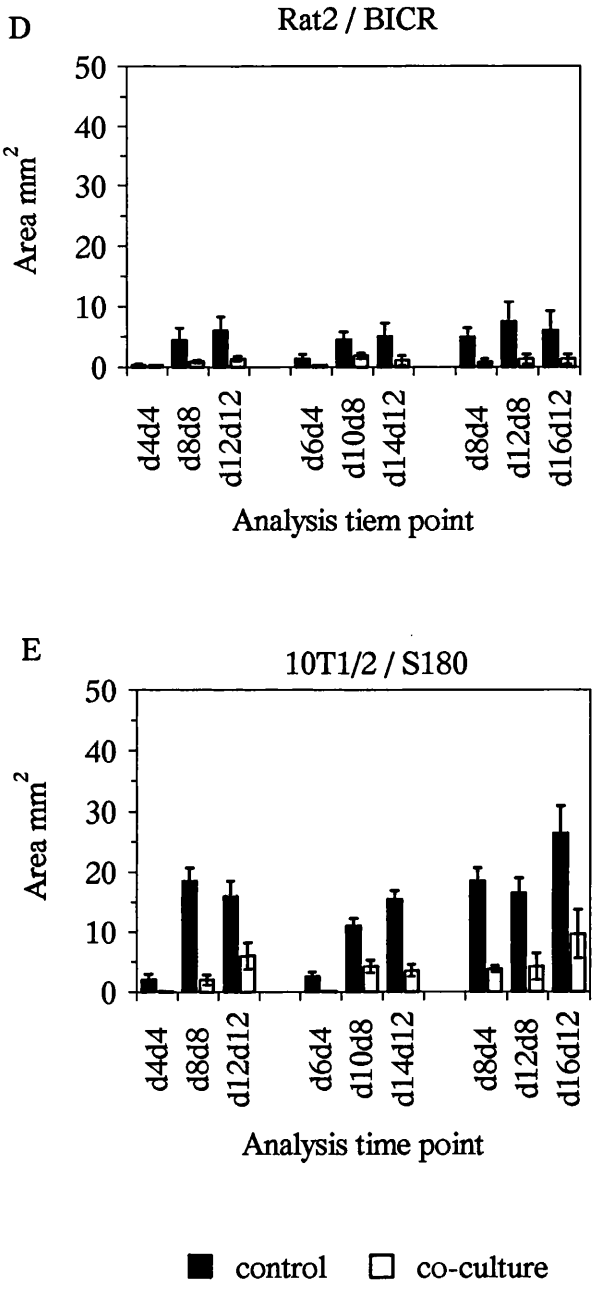


Figure 3.11. Continued: Average focus and colony size

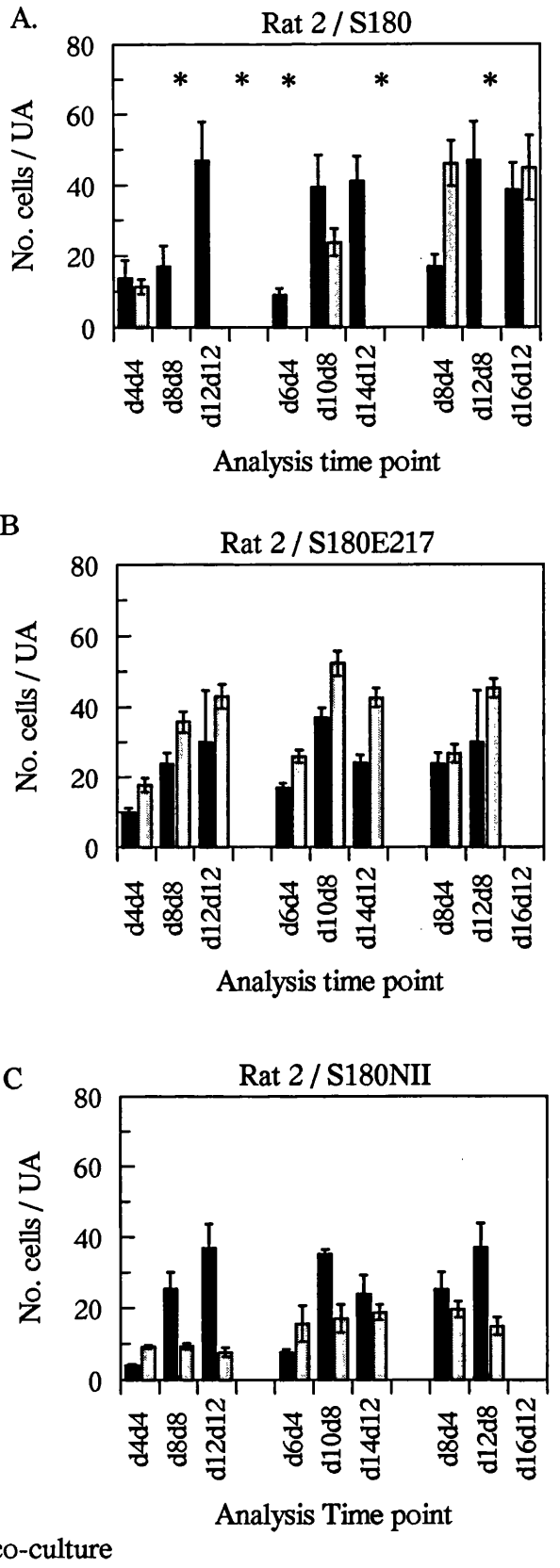


Figure 3.12. Cell density within foci and control colonies.

In co-cultures normal and transformed cells were plated at 10^6 and 500 cells / 90mm dish. Controls were represented by separate cultures of each cell lines seeded at the same densities as above. Cell density within foci and control colonies is given as the average number of cells per UA. For each time point 5 foci and 5 colonies were analysed. For each focus, cell density was determined from 10 UA's (or max. possible where focus/colony was small). The results from the 5 foci/colonies were averaged and plotted in the above graphs. Data missing in Rat2/S180 cultures (indicated by *) was due to high cell density, which prevented individual cells from being distinguished. Data missing at d16d12 in Rat2/S180NII and Rat2/S180E217 cultures is due to loss of cells during fixation. The S180 data has been used in Figures A & E.

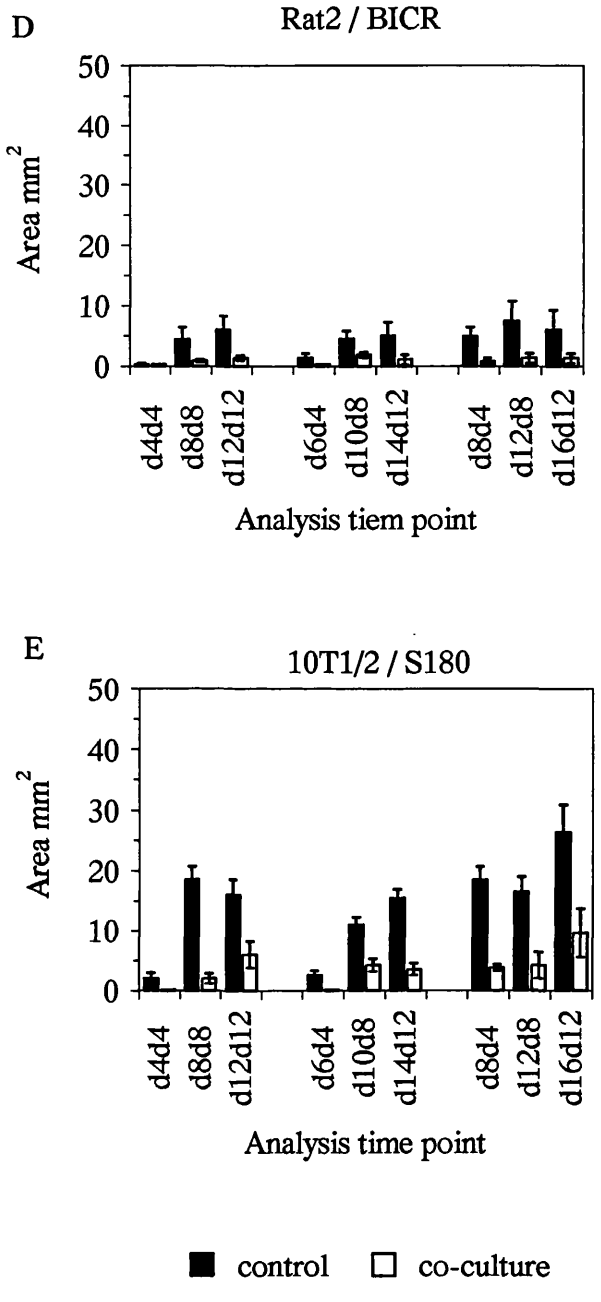


Figure 3.11. Continued: Average focus and colony size

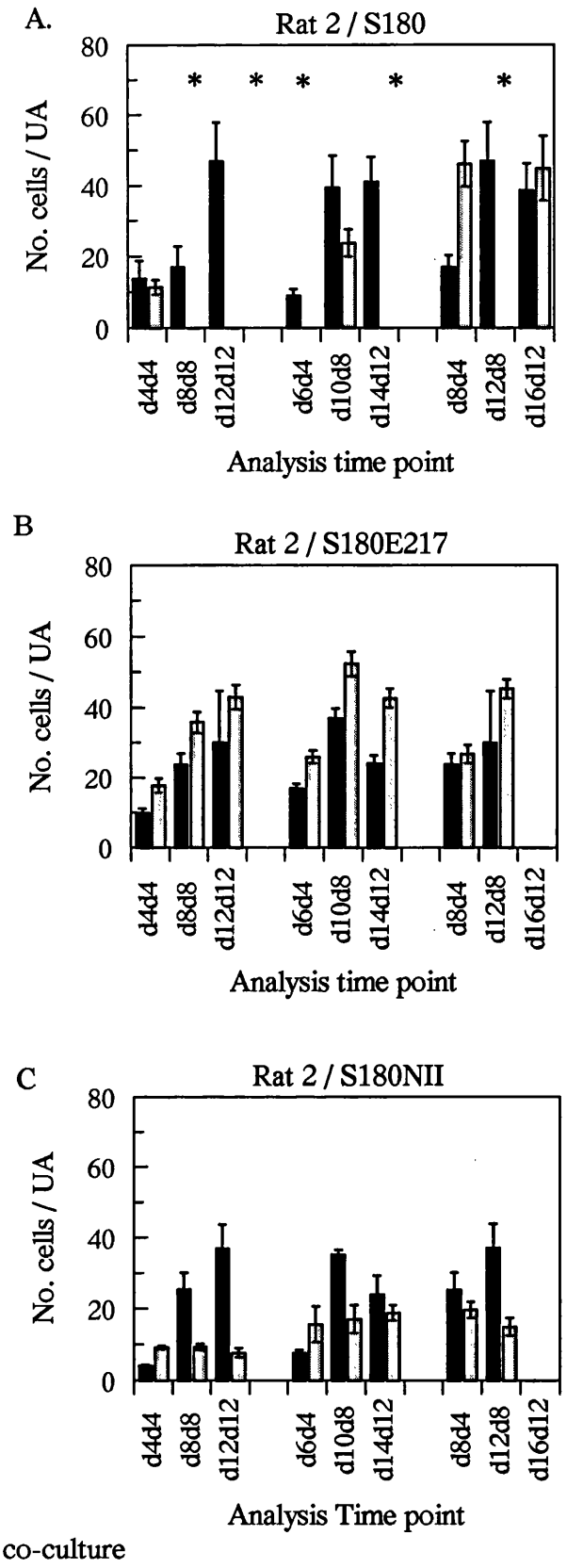


Figure 3.12. Cell density within foci and control colonies.

In co-cultures normal and transformed cells were plated at 10^6 and 500 cells / 90mm dish. Controls were represented by separate cultures of each cell lines seeded at the same densities as above. Cell density within foci and control colonies is given as the average number of cells per UA. For each time point 5 foci and 5 colonies were analysed. For each focus, cell density was determined from 10 UA's (or max. possible where focus/colony was small). The results from the 5 foci/colonies were averaged and plotted in the above graphs. Data missing in Rat2/S180 cultures (indicated by *) was due to high cell density, which prevented individual cells from being distinguished. Data missing at d16d12 in Rat2/S180NII and Rat2/S180E217 cultures is due to loss of cells during fixation. The S180 data has been used in Figures A & E.

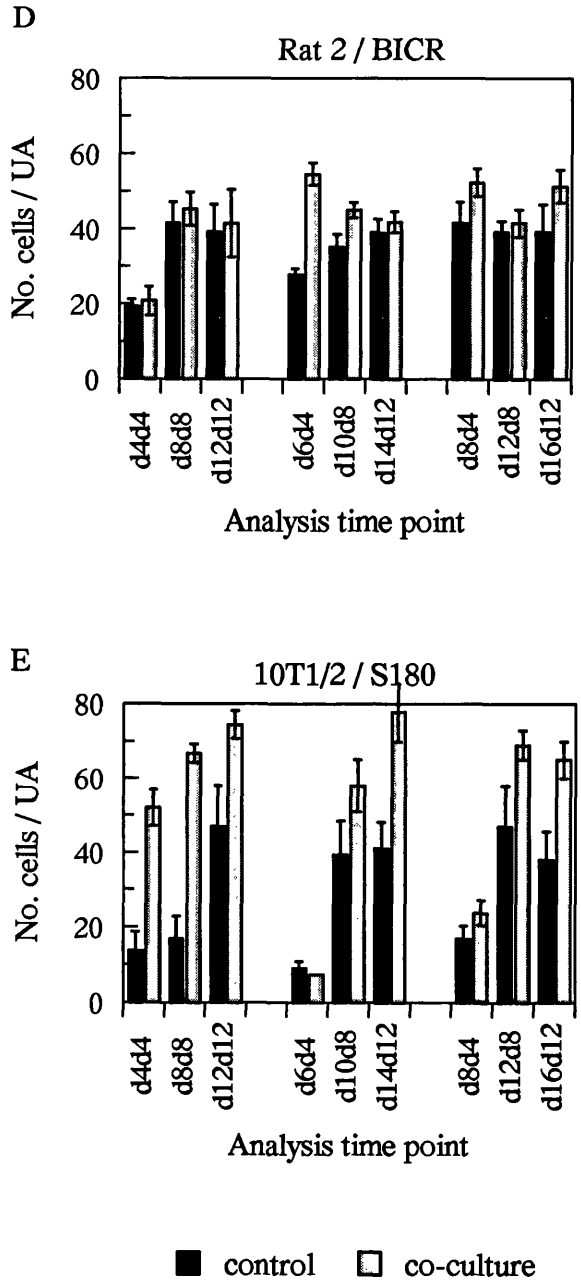


Figure 3.12. Continued: Cell density within foci and control colonies

Figure 3.13. Cell density within foci and control colonies.

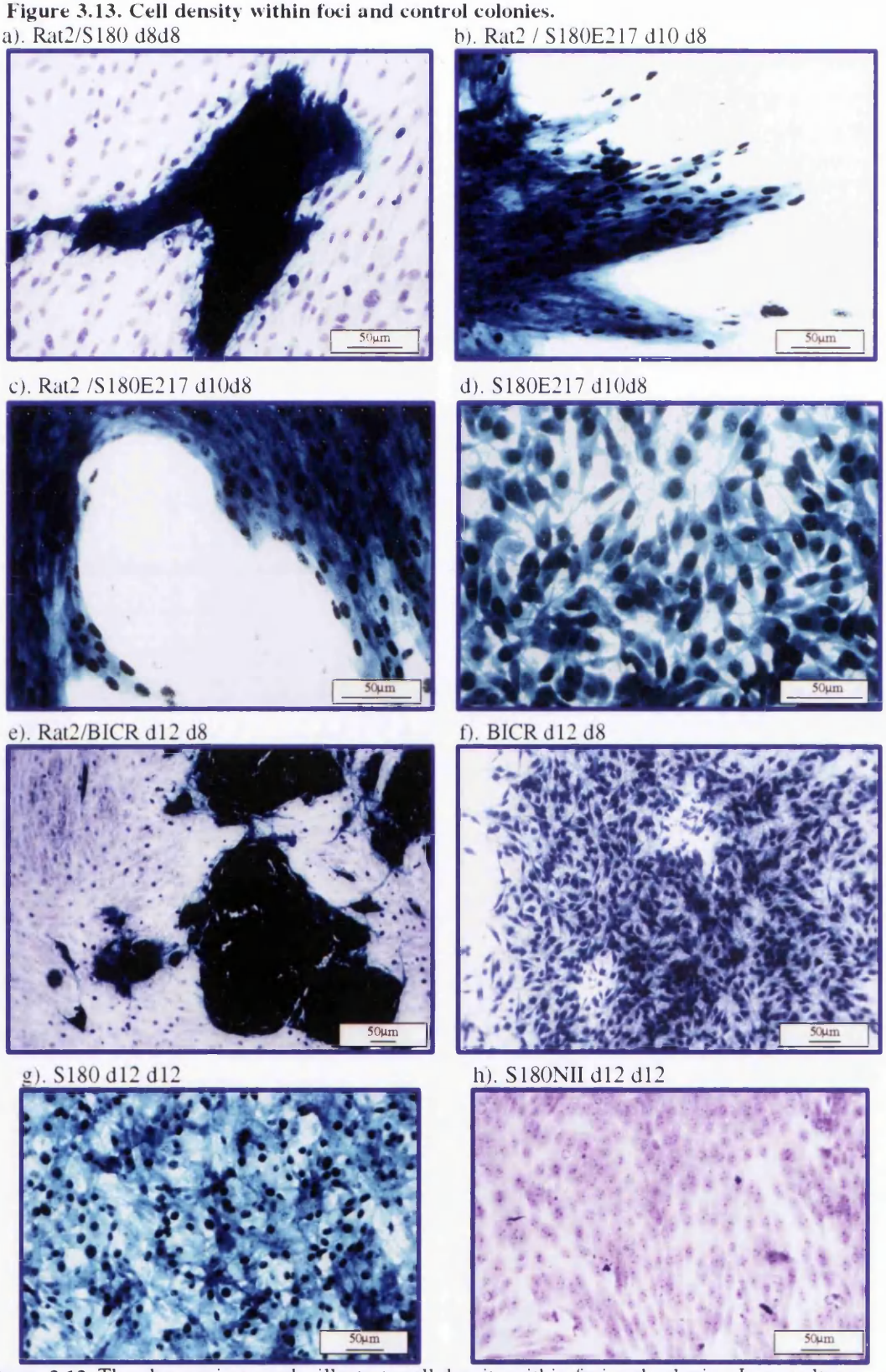


Figure 3.13. The above micrographs illustrate cell density within foci and colonies. In co-cultures normal and transformed cells were plated at 10^6 and 500 cells / dish respectively. In colony controls, cells were plated at 500 cells / dish. In Figure a) individual cells are difficult to distinguish due to high cell density and high levels of β-gal - which leads to a dark blue stain, however, in b), cells are also at high density but the level of staining still enables single cell identification. In Figures c) - f) cell density in transformed foci and respective control colonies is compared. In Figures g) & h) cell density in colonies of related cell lines is compared, for details see text. Bar represents scale.

3.4.3. Inhibition of cell division within the foci.

It is clear from the results in section 3.3 that focus formation assays provide only limited information on the inhibition phenomenon. Cell density analysis has shown that foci can be compressed by surrounding normal cells and this appears to contribute to the explanation of focus size suppression. This would imply that estimates of growth inhibition based only on focus size may be inaccurate. It is not yet clear what the actual level of growth within the foci is and until this is ascertained the role of GJIC can not be properly assessed. Only when these points are addressed can the questions detailed in the Introduction to the results (see section 3.1) be answered. The level of cell proliferation within the foci and control colonies has been measured using a growth assay based on [^{3}H]-thymidine incorporation and analysis by autoradiography.

Section 3.4.2 describes the experimental set up and the cell combinations used. At each time point the percentage of dividing transformed cells in control and co-cultures was obtained after analysing 5 colonies and 5 foci. For each focus/colony the average number of labelled and unlabelled cells/UA was obtained from 10 UA's (or max. possible when focus/colony was small). The results from the 5 foci/colonies were averaged and plotted as percentages in the graphs presented in Figure 3.14. The LI of the normal cells was measured in all co-cultures to confirm they had reached saturation density and were growth inhibited. The LI was obtained from counting the number of labelled and unlabelled cells in 20 UA's (samples were taken as far away as possible from any foci).

The average LI obtained for the normal cells over all time points was 3.7% ± 1.2 for Rat2 cells and 4.2% ± 1.4 for 10T1/2 cells. These figures indicate that the normal cells have reached their saturation density (section 3.1.1) and are significantly growth inhibited. However, growth of Rat2 cells (but not 10T1/2 cells) above the background level was observed close to many foci in all of the co-cultures and this has been analysed further in section 3.4.6.3.

S180 cells (in culture with Rat2 cells):

In control cultures of S180 cells, the LI within the control colonies drops considerably after 12 days in culture (Figure 3.14.A). This is consistent with earlier data (section 3.1) which shows that the growth of the S180 cell population begins to slow down after long periods in culture. However, this does not appear to be due to nutrient deprivation or saturation density. This decrease in cell proliferation corresponds with cell density levelling off over time (Figure 3.12.A). Analysis of cell growth in many of the S180 foci in co-culture with Rat2 cells was hindered by the high cell density and high intensity β-gal staining of the cells (as indicated by asterisks in Figure 3.14.A). Although individual cells within the foci could not be distinguished at all time points it was clear that proliferation was high, as indicated by the high level of labelling above the foci.

However, it can be seen in Figure 3.14.A. that inhibition was recorded at d10d8 and d8d4. There were also several examples of smaller than average foci present in many of the Rat2 /S180 co-cultures, (e.g. Figure 3.15.e). The proportion of proliferating cells in such foci was low, in Figure 3.15.e, for example only ~16% of cells are dividing whereas in respective control colonies the proliferation was close to 100 % - Figure 3.14.A.

S180E217 cells (in culture with Rat2 cells):

S180E217 cells in control colonies follow a similar labelling pattern over time as their parental S180 cells i.e. by day 12 their LI has significantly dropped (Figure 3.14.B). When the S180E217 cells are plated simultaneously with the Rat2 cells and a focus diameter cut-off point of 0.1mm (area: 0.008mm^2) is used, there is little inhibition of focus number (section 3.3.5). However, the size difference between the foci and respective colonies is marked (e.g. at d12d12 average colony size is 10mm^2 & average focus size, formed on a background of Rat2 cells is 0.5mm^2; Table 3.10.b). This would suggest that there is a high level of growth inhibition occurring. However, analysis of the foci which do form at these time points, reveals that cell growth is not significantly suppressed (Figure 3.14.B). Similarly, when S180E217 cells are pre-established for 2 or 4 days before the normal cells are added, there is very little, if any, cell growth inhibition in the subsequent foci (e.g. Figure 3.13.c).

Although cell density within these foci is higher than in control colonies it is clearly not sufficient to account for the difference in size between the foci and the control colonies (e.g. Figure 3.10.a). This would suggest that a high proportion of cells within the foci are unable to attach to the culture dish (due to high cell density) and are therefore pushed off the dish.

S180NII cells (in culture with Rat2 cells):

The level of cell proliferation in control cultures of S180NII cells remains high in the majority of time points analysed (Figure 3.14.C & for example Figure 3.15.a). The level of proliferation in these colonies is consistent with an increase in colony size and colony density over time (Figure 3.11.C & Figure 3.12.C respectively).

When S180NII cells are co-cultured with Rat2 cells the highest level of inhibition occurs when the two cell types are plated simultaneously (Figure 3.14.C) and examples of inhibited foci can be seen in Figure 3.15. b & c. However, by the 12th day of the co-culture, a small proportion of cells within many of the S180NII foci are beginning to escape the growth suppression (Figure 3.14.C. e.g. Figure 3.15.d). This is consistent with a small increase in their average focus size (Figure 3.11.C). Foci formed from S180NII cells which were pre-established for 2 and 4 days have a higher LI than foci formed when the two cell types were plated simultaneously, suggesting that when a focus has grown to a certain size (>0.1mm^2) there response to the inhibitory mechanism is markedly reduced. However, unlike S180E217 cells, S180NII cells are still

significantly suppressed (30-60% inhibition; Figure 3.14 C). There appeared to be no pattern to the distribution of inhibited cells within the S180NII foci, i.e. cells at the edge of the focus appeared to be no more likely to be inhibited than cells at the centre.

BICR cells (in culture with Rat2 cells):

BICR cells are well coupled to Rat2 cells but only show a very low level of growth inhibition when co-cultured with them (Figure 3.14.D). This low level of growth inhibition is maintained even when the BICR cells are pre-established. However, the difference in size between foci and respective colonies recorded (see Figure 3.14.D) was large, which would suggest that increased focus cell density significantly contributes to the explanation of focus size inhibition or cells are lost from the culture dish due to overcrowding.

S180 cells (in culture with 10T1/2 cells):

The number of foci formed by S180 cells in co-culture with 10T1/2 cells was greater than the number of colonies formed in control cultures and would appear to show that some form of feeder effect was taking place (section 3.3.2). However, the cell proliferation data (Figure 3.14.E) shows that a significant, but generally low level of inhibition is imposed on the S180 cells at most time points, even when the S180 cells are pre-established. It is possible that the feeder effect may occur before the 10T1/2 cells reach their saturation density and therefore before any inhibition is imposed on the S180 cells. Subsequent foci show low levels of growth suppression which together with increased cell density (section 3.4.2) leads to smaller foci in comparison to controls. It is not clear what causes the reduction in the growth of the S180 cells. Earlier results (section 3.1) would suggest that as the age of the culture increases the level of proliferation decreases but these changes are not associated with a saturation density or nutrient deprivation. Many smaller than average foci were observed in the 10T1/2 /S180 co-cultures (e.g. Figure 3.15.f). The level of cell proliferation in these foci is generally low in comparison to control colonies and the average size foci. It is unlikely that the inhibition is mediated by GJIC since the two cell lines do not appear to communicate with each other (Table 3.3).

3.4.4. Summary.

The cell proliferation analysis would appear to show that inhibition phenomenon is not as common as the literature tends to suggest (Section 1.6). Focus size measurements are presented in many published reports as indications of growth suppression, however, it has been shown here that focus compression (as indicated by increased focus cell density) can contribute significantly to the difference in size between

the foci and control colonies. Furthermore, detailed examination shows that the proportion of cells dividing within many compressed foci can often be very high.

The results from the cell proliferation analysis have added further complexity to the understanding of the role of gap junctional communication in the inhibition pathway. Transformed cell lines which are well coupled to normal cell lines may not necessarily be suppressed in co-culture with them. In this study for example, BICR cells, which are coupled to the Rat2 cells, are poorly growth inhibited by them. This would suggest that these cells do not respond to the putative inhibition factors transmitted from the normal cells and/or that the growth control mechanisms impaired in this cell line are not associated with loss of GJIC. 10T1/2 and S180 cells do not appear to communicate with each other, yet when they are cultured together a low level of S180 growth inhibition is recorded. And in some instances several small, highly inhibited foci were observed. However, communication between the two cell lines may be very low and therefore go undetected with the type of communication assay used here. Over long periods of time, transfer of putative inhibitory factors may be sufficient to cause low level growth suppression.

It has been shown that transfection of the poorly coupled S180 cells with E or N cadherin results in increased homologous and heterologous communication (section 3.2.2). However, only S180NII cells are growth inhibited by the Rat2 cells. The S180E217 cells appear to remain in cell cycle in the presence of the Rat2 cells although the difference in size between the foci and control colonies is sometimes very large. This would suggest that the physical presence of the surrounding normal cells (which results in high focus cell density) may cause cells within the foci to detach due to overcrowding. It is not clear why N cadherin but not E cadherin expression can confer suppressible phenotypes on the S180 cells. Both cell lines show increased communication relative to parental S180 cells it would appear that GJIC is necessary but not sufficient for inhibition to occur. It is possible that adhesion, rather than GJIC, is important for suppression and the increase in GJIC is brought about by the increase in adhesion. However, it is not clear why N cadherin, but not, it would seem, E cadherin alters the interactions between S180 and Rat2 cells to one which favours suppression.

The size of the initial transformed cell colony appears to be important in terms of the level of growth inhibition which is imposed on the S180NII foci. When the S180NII cells are plated simultaneously with the Rat2 cells they are able to go through 2-3 cell divisions before the Rat2 cells reach saturation density and become growth inhibited. Most of the colonies over the first 12 days of co-culture (when plated simultaneously with the normal cells) remain below the 0.1mm diameter threshold size and are normally ignored. Of those S180NII foci which have reached the threshold size, a high proportion of cells are still inhibited. However, when the S180NII cells are pre-established the foci show increased levels of cell proliferation. These data suggest that the larger the initial colony size the less likely the cells are to be inhibited by surrounding normal cells. The

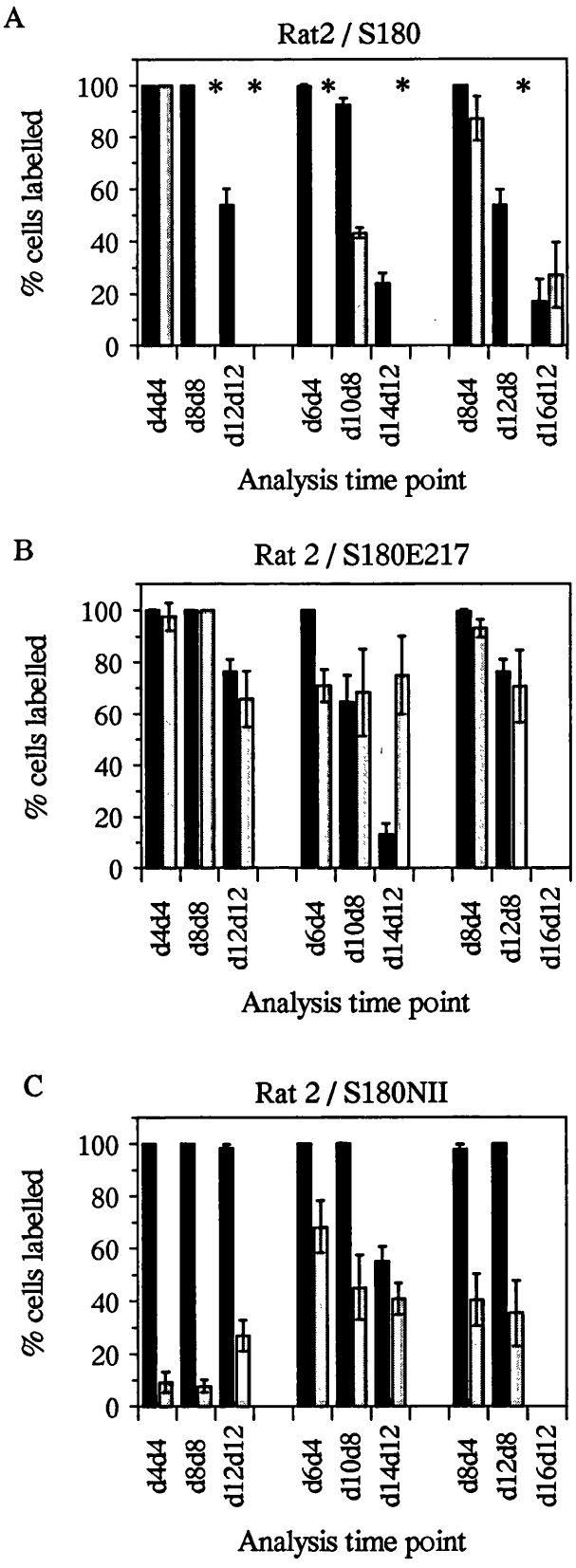


Figure 3.14. Labelling index of transformed cells within colonies and foci

In co-cultures normal and transformed cells were plated at 10^6 and 500 cells / 90mm dish respectively. Controls were represented by separate cultures of each cell line seeded at the same densities as above. For each time point 5 colonies and 5 foci were analysed. For each colony/focus the average number of labelled & non-labelled cells/UA was determined from 10 UA's (or max. possible when foci/colonies were small). The results from the 5 foci/colonies were averaged and plotted as percentage labelled cells in the above graphs. The asterisk indicates that focus density was too high to be able to distinguish individual cells. No data could be obtained in Rat2/S180NII or Rat2/S180E217 cultures at the time point d16d12 because cultures detached during fixation.

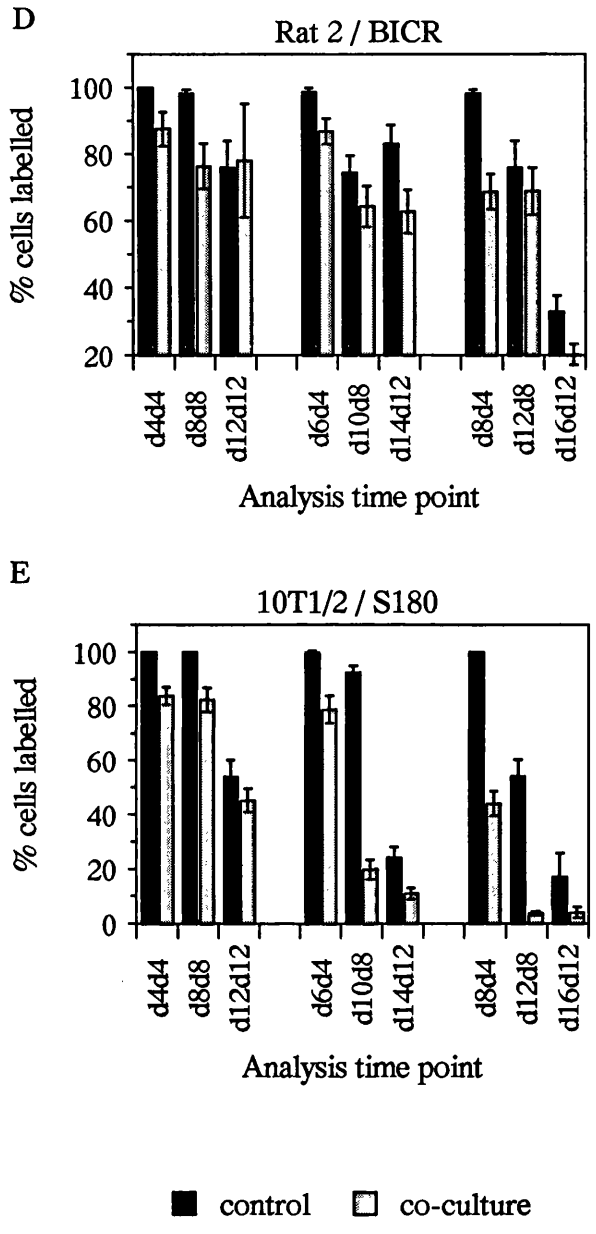
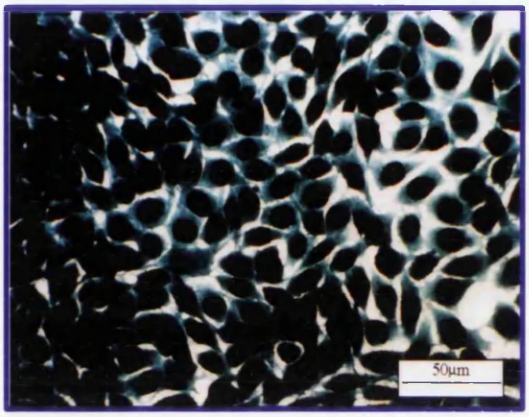


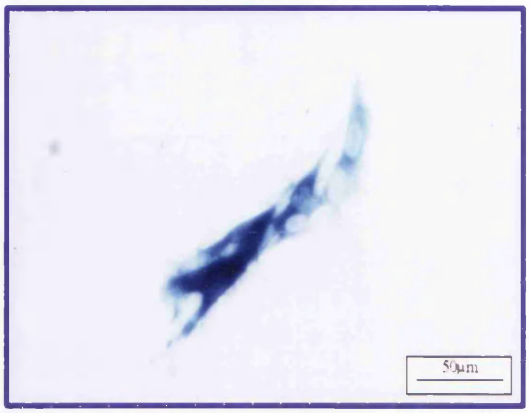
Figure 3.14. Continued: Labelling index of transformed cells within colonies and foci

Figure 3.15. Levels of proliferation within foci and control colonies

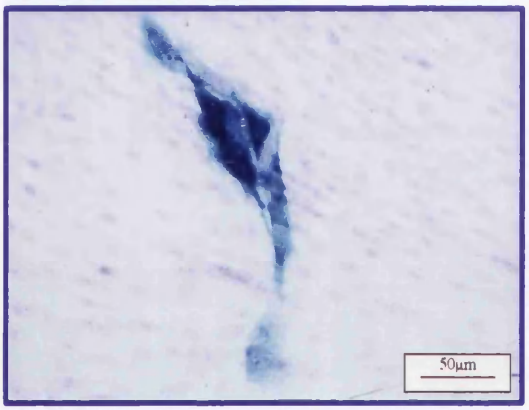
a). S180NII d8d8 control



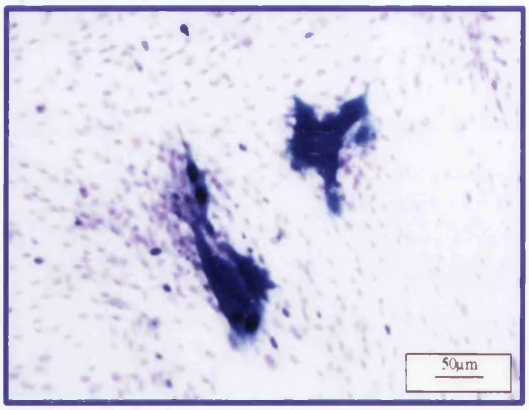
b). Rat2 / S180NII d8d8



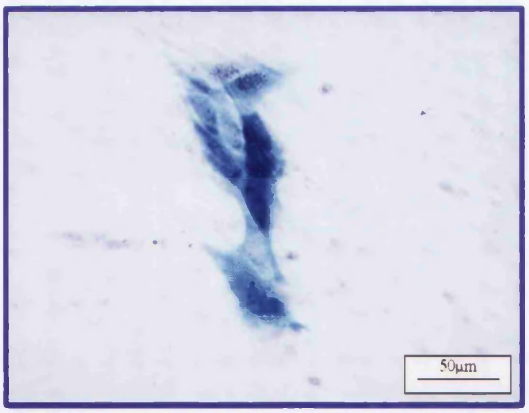
c). Rat2/S180NII d8d8



d). Rat2 / S180NII d12d12



e). Rat2 / S180 d8d4



f). 10T1/2 /S180 d8d4

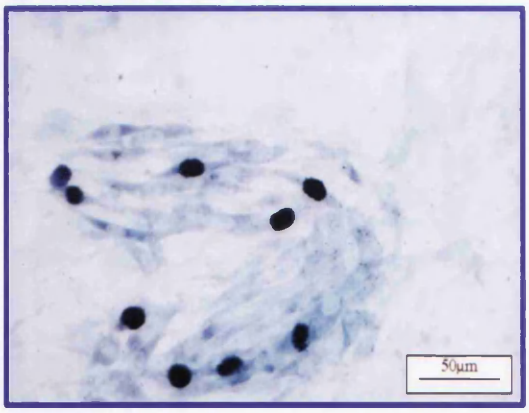


Figure 3.15. The above figures are examples from proliferation assays designed to study the effect of excess normal cells on the growth of transformed cells. Normal and transformed cells were plated at 10^6 and 500 cells/ dish respectively. Controls were represented by separate cultures of each cell line at the density as above. Figure a) shows the typical level of proliferation within a transformed cell colony (S180NII in this example) at the time point shown. Figures b) & c) show S180NII cells cultured with excess Rat2 cells for 8 days. Note the high level of growth inhibition. In d) it can be seen that after 12days culture with the Rat2 cells, S180NII cells are begining to escape suppression. In most instances S180 parental cells appeared to escape suppression, however, smaller than average S180 foci were observed in Rat2 and 10T1/2 co-cultures, such as those shown in Figures e) & f). However, not all cells within these foci were growth inhibited. Bar represents scale.

size of colony which can escape growth inhibition may be determined by the level of GJIC between the transformed and surrounding normal cells. For example S180NII cells show higher heterologous communication than S180E217 cells. The rate of inhibition transfer may therefore be important, coupled with the ability of the transformed cells to maintain their aberrant phenotype (possibly by the production of stimulatory growth factors), which increases as the focus grows in size.

3.4.5. The effect of the normal cell monolayer on focus morphology.

It has been shown throughout previous sections that the physical presence of the normal cells appears to restrict focus expansion as indicated by higher cell density in many foci. However, transformed cell proliferation remained high in most of the cultures which led to marked increases in the size of many foci over time. The morphology of these growing foci appears to be influenced by the surrounding normal cell monolayer. Examination of focus and cell morphology may provide information on whether the expanding foci grow over the surrounding monolayer or whether the normal cells act as a barrier to focus expansion and are pushed back as the foci expand.

The morphology of the foci and colonies, whose growth was analysed in section 3.4.3, have been examined. S180 and BICR control colonies tend to be circular in shape with ragged edges. The edges of S180E217 and S180NII colonies tended to be smoother because these cells are less inclined to overlap (section 3.4.2; Figure 3.13. g & h) and this is likely to be caused by better cell-cell adhesion. The majority of foci had uneven edges which is probably generated by the ability of the cells at the periphery of the colony to move into areas clear of other cells. Cell density at the periphery of the colonies is generally less than in the centre where competition for space on the culture dish is likely to be greater and where multi-layering is more common (e.g. Figure 3.16.a & b).

Different transformed cells formed foci with different morphologies, however, all appeared to be influenced by the presence of the normal cells. Focus morphology also varied depending on the length of time the foci spent in co-culture with normal cells and whether the transformed cells were pre-established.

In general, foci formed by BICR cells (when plated simultaneously with the Rat2 cells) tended to be circular (e.g. Figure 3.16.c) whereas foci formed by S180 cells or the S180-cadherin transfectants, when the cells had been plated simultaneously with the Rat2 cells, tended to align with the 'grain' of the normal cell monolayer and were generally longer and thinner (e.g. Figure 3.16.d). In the majority of instances the foci formed by all of the transformed cells tended to have smoother edges than the control colonies.

Foci formed from pre-established transformed cells varied in morphology from foci formed when both cell types were plated simultaneously. When the normal cells

were added to pre-established colonies of BICR, S180 or S180-derived cells, the subsequent foci initially had ragged edges (e.g. Figure 3.16.e). This would suggest that the normal cells were able to move and divide in-between the transformed cells at the focus periphery where cell density was initially lower. However, as the culture period increased and the transformed cells continued to divide, the average focus cell density in the majority of instances also increased and often to a much greater extent than control colonies (Figure 3.12). This would suggest that the normal cells, upon reaching confluence, compete with the transformed cells for space on the culture dish, thereby reducing the rate of focus outgrowth (leading to increased cell compression, multilayering and consequently an increase in focus cell density). In many instances, particularly in later time points, the edges of most foci of all cell types became smoother although, in general, the pre-established foci remained more irregularly shaped (e.g. Figure 3.16.f).

These observations would also suggest that there is little transformed-cell over growth of the normal cell monolayer. That is, within the majority of foci, cell multilayering / overlapping is frequent, yet cells at the focus periphery rarely project over the 'grain' of the monolayer (e.g. Figure 3.16. c). In Figures 3.16. g, h & i, for example, it can be seen that transformed cell processes rarely project across the 'grain' of the monolayer, but do project into it when they are oriented with the 'grain' of the monolayer. This would suggest that the transformed cells can grow more easily in-between the normal cells but not across them. One common feature of normal cells is that they generally do not show nuclear overlap (but can show cytoplasmic overlap). In general the transformed cells also appear to avoid nuclear overlap with the normal cells (though not with themselves), for example, Figures 3.16. g, h & j (red arrows). Clearly, in some instances the transformed cells do grow over the normal cells and this has been highlighted in Figure 3.16. h (green arrows).

It would appear that the transformed cells avoid, or are partially prevented from growing over the monolayer. However, despite the apparent competition for space on the culture dish many foci continue to expand. If, as the focus and cell morphology analysis suggests, there is generally little transformed-cell overgrowth of the surrounding monolayer then an increase in normal cell density would be expected as the foci expand and push back the normal cells. The increase in normal cell density is likely to be exacerbated further by the apparent growth stimulation of normal cells which surround many foci (e.g. Figure 3.16. c & d - yellow arrows; this phenomenon has been examined further in section 3.4.6.3).

The question therefore remains as to what happens to the normal cells which lose space on the culture dish to the dividing transformed cells. This is addressed next.

Figure 3.16. The effect of normal cells on focus morphology.

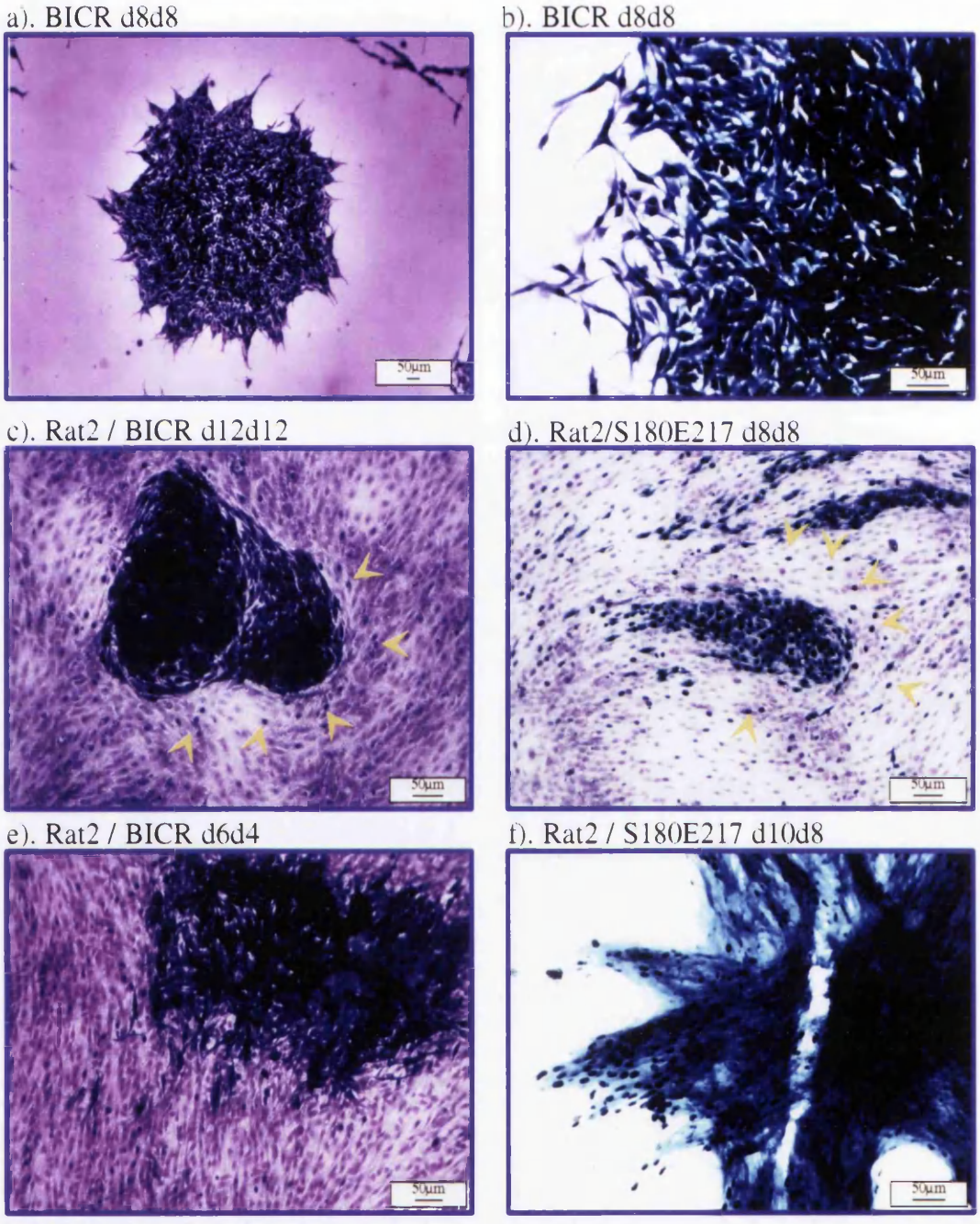


Figure 3.16. The micrographs above show the effect of normal cells on focus morphology. See Figure 3.14 for experimental details. Cell lines and cell combinations used, together with culture times are given above each figure. A BICR colony is shown in a) which in terms of shape and a ragged periphery is typical of most transformed cell colonies. In b) it can be seen that the cells at the periphery of the colony are at lower density than at the centre. In c) the BICR focus shown has smoother edges than the BICR colony but maintains a circular shape. The cells of the foci formed by S180 or S180-derived cells were generally aligned with the grain of the surrounding monolayer, foci were longer than those formed by BICR cells but also had smooth edges, e.g. Figure d). The effect of pre-establishing the transformed cells on subsequent focus morphology can be seen in Figures e) & f). In general foci begin with ragged edges, eg. Figure e), but over time the edges of the foci become smoother leading to irregularly shaped foci, eg. Figure f). Yellow arrows indicate examples of growth stimulated normal cells - a phenomenon which has been analysed further in section 3.4.3.2. Bar represents scale.

Figure 3.16. Continued: The effect of normal cells on focus morphology

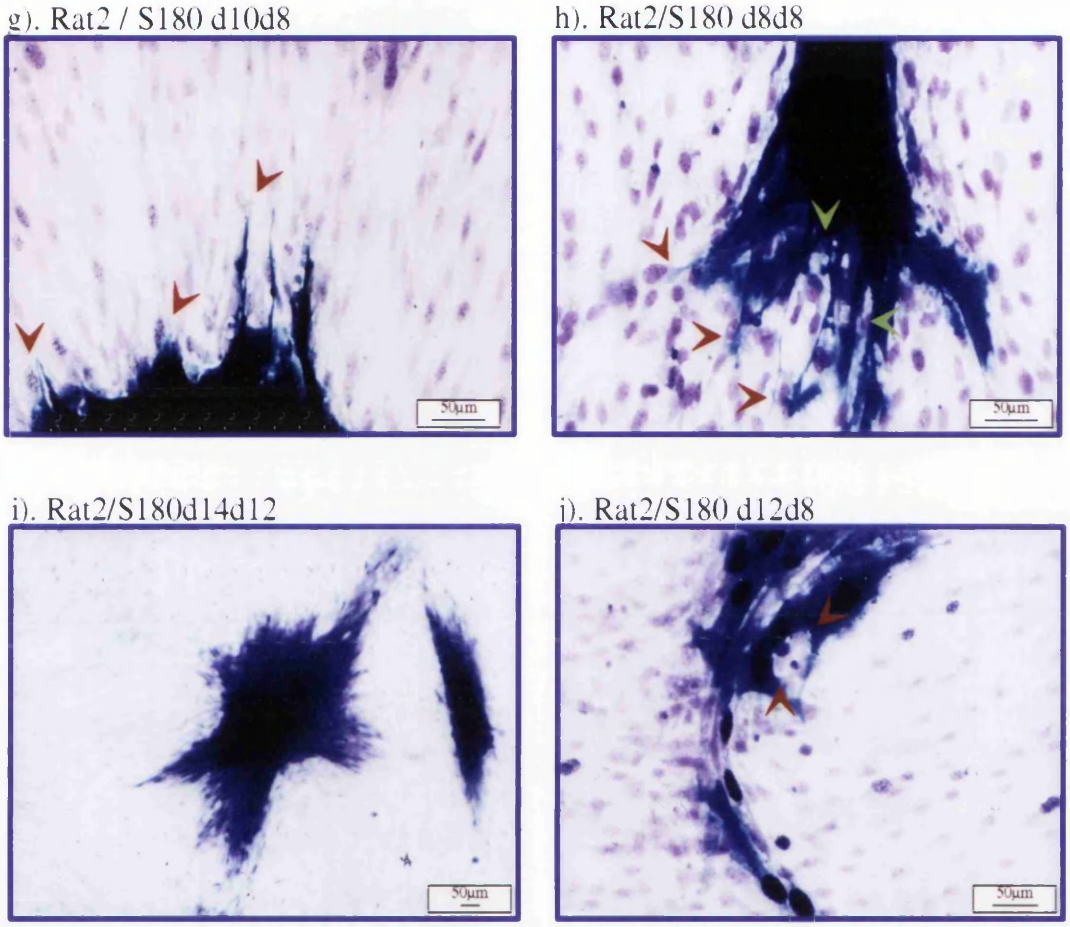


Figure 3.16: Continued. Cell lines and cell combinations used, together with culture times are given above each figure. Red arrows indicate examples where the transformed cells appear to have avoided growing over the nuclei of normal cells. Green arrows indicate examples where the transformed cells appear to overlap the nuclei of normal cells. Bar represents scale.

3.4.6. The effect of transformed cells on surrounding normal cells.

It would appear from the focus morphology analysis that, in general, the transformed cells do not grow over the normal cells. However, there is continued transformed cell growth in many of the co-cultures and marked increases in the size of some foci in some of the cultures. For example after 12 days in co-culture with Rat2 cells the S180NII foci take up ~0.2% of the culture dish and S180E217 foci take up ~12% of the culture dish (these approximate figures are based on the *total number of foci* x *the average area of foci;* obtained from data in Tables 3.10.a & b).

Since there appears to be little overgrowth of the two cell types it is not clear what happens to the normal cells as the foci expand. An increase in normal cell density, particularly around the focus periphery where demand for extra space is presumably high, would be expected. This is likely to be exacerbated by the apparent small increase in normal cell proliferation surrounding many of the foci. The possibilities as to how the normal cells are accommodated when the foci expand are listed below:

1. As normal cells lose space on the culture dish to the expanding foci, they may shuffle up. The increase in density may be dissipated over the surface area of the culture dish which, over time, may lead to a general increase in normal cell density over the entire dish.
2. The normal cells may be physically pushed off the plate by the expanding foci or apoptose (possibly as a result of a signal from the transformed cells or as a result of over crowding).

These possibilities have been examined and the results presented in the following section.

3.4.6.1. *The effect of expanding foci on normal-cell density.*

To determine if normal cell density increases next to expanding foci and over the monolayer in general, normal-cell density (cells per UA) was determined within 0.175mm of the focus periphery and as far away as possible from any foci. Cell density was also measured in control cultures (10^6 cells / 90mm dish) at all time points. For each time point the cell density in the control cultures was obtained from 20 sample UA's and the results presented in Figure 3.17. In co-cultures, for each time point, cell density was determined next to 5 foci. Cell density was calculated from 10 Sample UA's around each focus (or max. possible where foci were small). The results from the 5 foci were then averaged and plotted in the graphs presented in Figure 3.18. Cell density as far away as possible from any foci at each time point was obtained from 20 sample UA's and the results plotted with the cell density data obtained next to the foci in Figure 3.18.

Rat2 cell density directly adjacent to either S180, S180E217 or S180NII foci is not significantly different (P>5%) from cell density away from the foci (Figure 3.18. A, B & C; e.g. Figure 3.19.b). However, in general cell density next to the foci is slightly

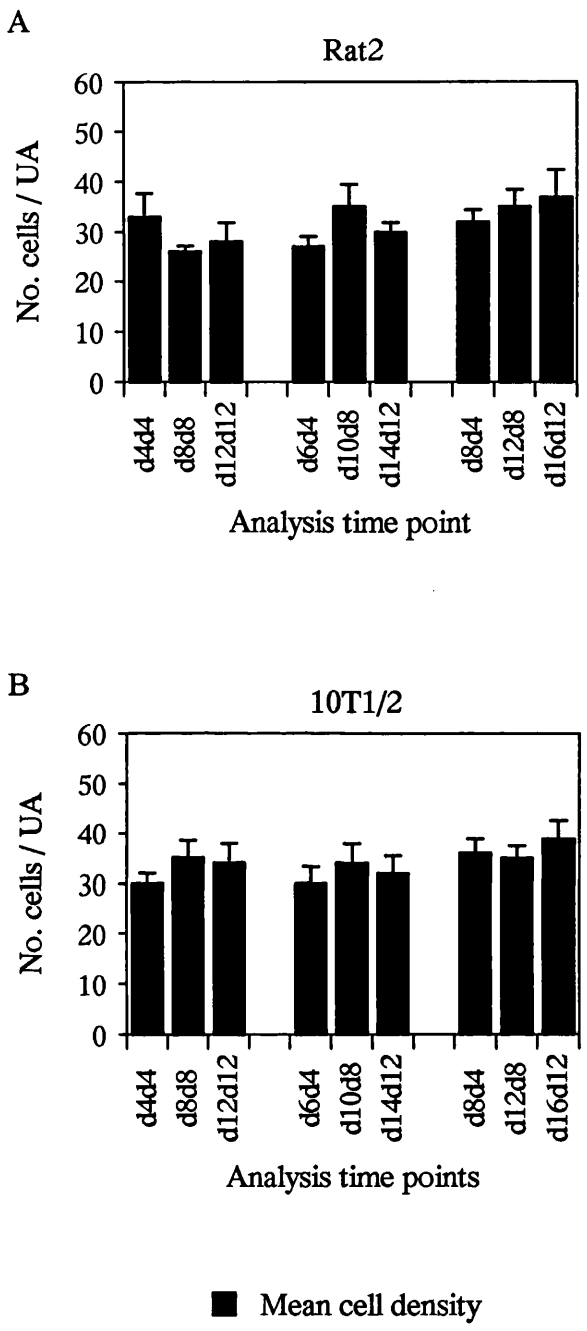


Figure 3.17. Cell density in separate cultures of Rat2 and 10T1/2 cells.

Separate cultures of Rat2 and 10T1/2 cells were plated at 10^6 cells per 90mm dish. For each time point the average number of cells per UA was determined from 20 sample UA's.

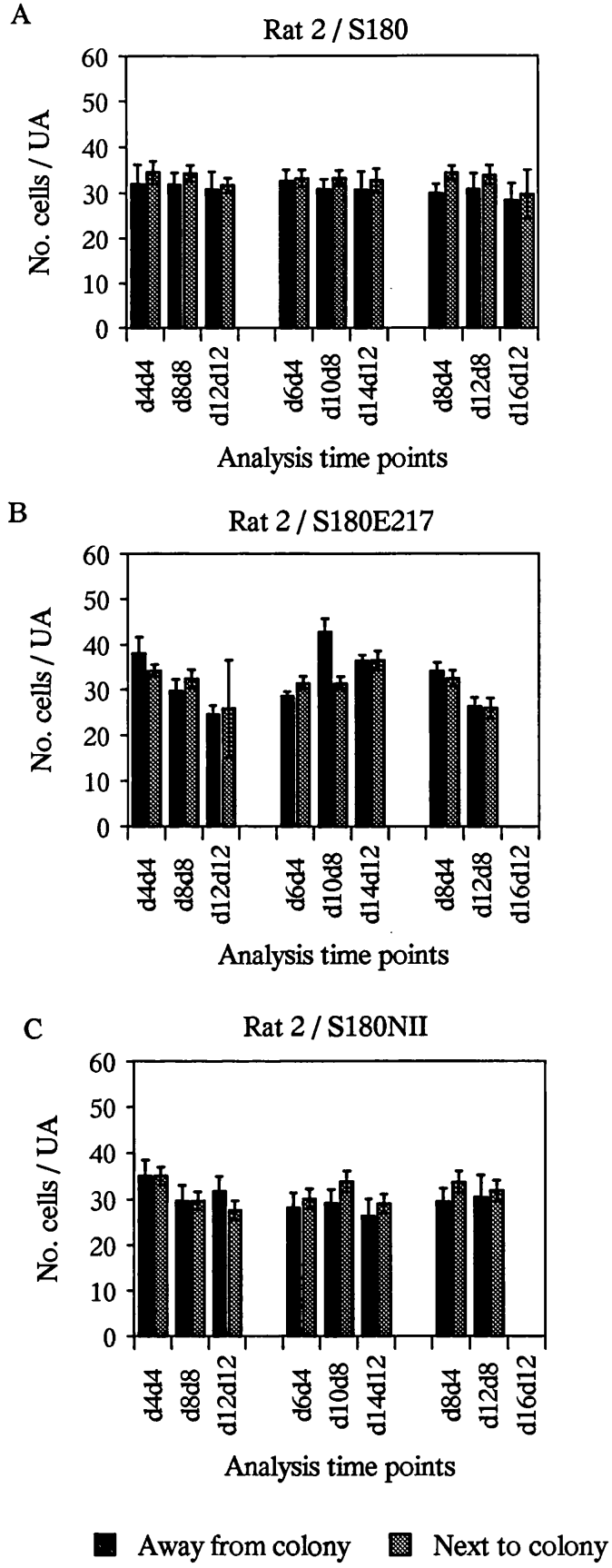


Figure 3.18. Normal cell density next to and away from the focus periphery.

Normal and transformed cells were plated at 10^6 and 500 cells / 90mm dish respectively. For each time point normal cell density (average number cells / UA) was determined next to 5 foci. Normal-cell density was calculated from 10 sample UA's taken around the focus. The results from the 5 foci were averaged and plotted in the graphs above. Normal cell density was also taken as far away as possible from any foci. For each time point cell density was calculated from 20 sample UA's. Data was not obtained at d16d12 in Rat2/S180E217 & Rat2/ S180NII co-cultures due to loss of cells during fixation.

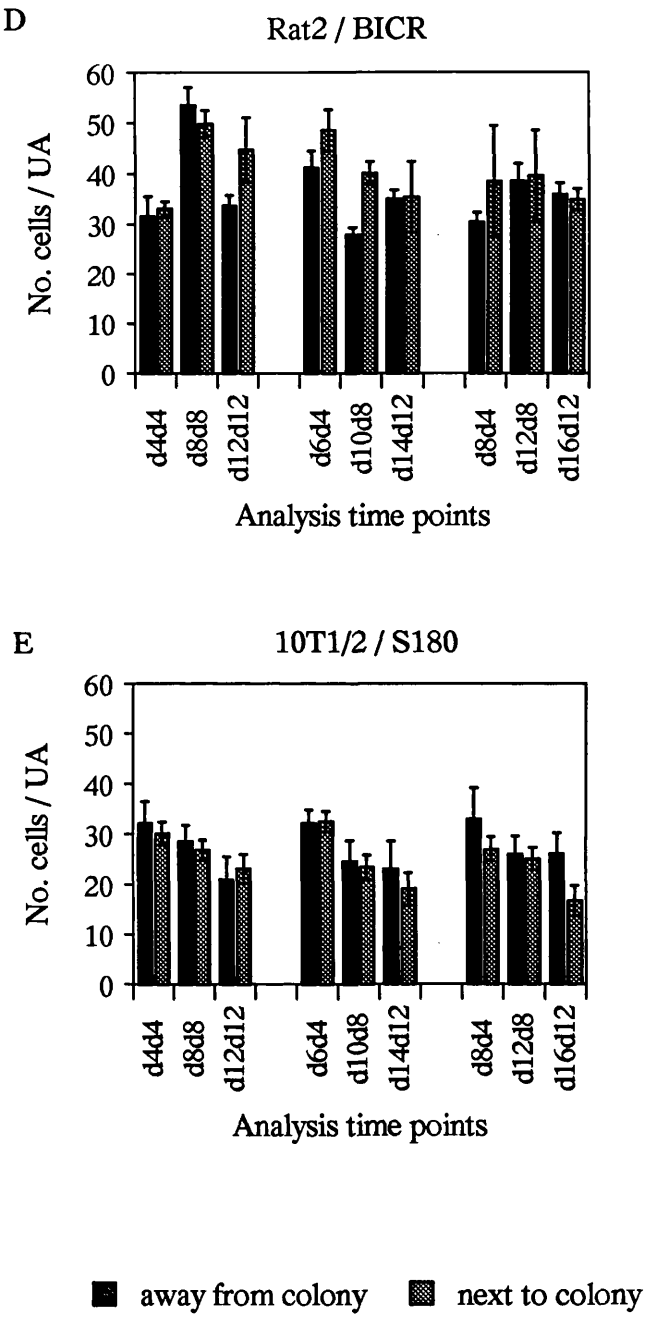


Figure 3.18. Continued: Normal cell density next to and away from the focus periphery

Figure 3.19. The effect of transformed cells on normal cell density and morphology.

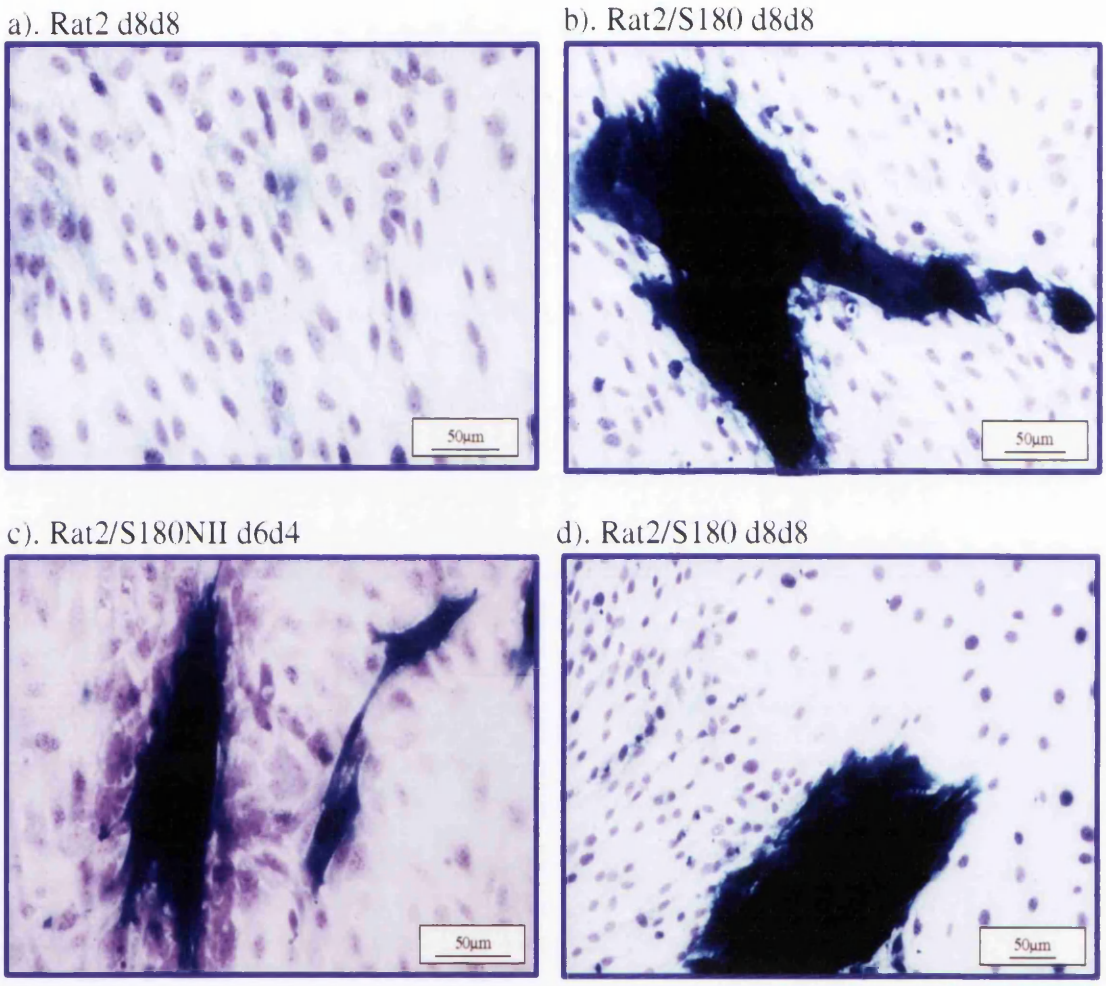


Figure 3.19. The examples above show the effect transformed foci have on normal cell density. The transformed cells were plated at 500cells/dish and the normal cells at 10^6/dish. The period of time spent in culture is given above each micrograph. Cultures were fixed in 1% Formaldehyde and 0.1% Gluteraldehyde in PBS for 30mins. They were then washed with PBS and stained with x-gal for 8hrs and Giemsa (1:10) for 10mins (section 2.5.1). In Figure a) cell density in a control culture of Rat2 cells is shown. In Figure b) normal cell density appears unaffected by the presence of the compact focus. However, Rat2 cells directly adjacent to the focus are generally thinner than cells away from the focus periphery. In Figure c), again, normal cell density appears unaffected by the compact focus, however, general variations in normal cell density throughout the monolayer can be observed to the left of the focus. In Figure d) a local increase in Rat2 cell density can be seen next to the S180NII foci. Bar represents scale.

higher (though not significantly) than cell density away from the periphery of the foci. In the few exceptions where there was an obvious increase in normal cell density around the focus periphery, it was very localised, e.g. Figure 3.19.c. However, such increases in cell density occurred infrequently and not in any particular cell combination. Within most of the co-cultures there were areas of the monolayer in which cell density varied from the average (e.g. to the left of the focus shown in Figure 3.19.d), however, these variations were not always associated with the foci and were also observed in many of the control cultures.

In Rat2 / BICR co-cultures (Figure 3.18.D) the density of the normal cells shows considerable variability in comparison to control cultures over many of the time points. It is not clear why such variability occurred with this particular cell combination, however, there appears to be no specific trends to the changes in density over time.

In 10T1/2 / S180 co-cultures (Figure 3.18.E) the general trend appears to be a reduction in normal cell density over time. This is perhaps due to cell death as a result of the ageing of the media (section 3.3) and unknown factors.

It can be concluded from the data presented in this section that there appears to be an insufficient increase in the normal-cell density around the foci over time, even when foci grow relatively large, to account for space on the dish lost to the expanding foci. Furthermore, expanding foci do not appear to cause a significant increase in the overall density of the culture.

3.4.6.2. *Apoptosis in control and co-cultures.*

Loss of normal cells due to apoptosis may account for the general absence of increased cell density throughout the monolayer. Furthermore, apoptosis may indirectly cause the normal-cell growth stimulation which was observed surrounding the periphery of many foci i.e. as the cells die the subsequent space may signal other normal cells to divide, which must then compete with the transformed cells for the available space on the culture dish.

To examine this possibility immunocytochemistry was used to detect apoptosis-induced DNA strand breaks (section 2.1.2). Focus assays were set up using co-cultures of Rat2 and S180E217 cells (foci formed by this cell lines are not growth suppressed and show marked increases in focus size over time). Rat2 cells were seeded at 10^6 / 90mm dish and S180E217 cells plated at 500 / 90mm dish either simultaneously with the normal cells or pre-established for 4 days. After 4 days the co-cultures were fixed in fresh 4% paraformaldehyde for 30mins and DNA strand breaks visualised, as described in section 2.6. Controls were represented by separate cultures of Rat2 cells, (plated at 10^6 / 90mm dish) fixed after the same time periods as the co-cultures. As positive controls, parallel cultures were treated with DNase I for 10mins (the procedure is described in section

2.6). As negative controls, fixed cultures of Rat2 cells were only treated with the TUNEL reaction solution (see section 2.6)

Low levels of apoptosis were found throughout the general population of Rat2 control cultures (Figure 3.20.c). The space vacated by the dead cells may signal other cells to divide. And the proportion of cells apoptosing per plate is would appear to account for the residual labelling index of 2-4 %, recorded in these cultures at saturation density (section 3.1.1). A similar level of Rat2 cell apoptosis was observed in co-cultures of Rat2 / S180E217 cells (Figure 3.20.d). There was, however, no specific localised apoptosis of normal cells surrounding the foci (Figure 3.20.e).

The normal cells may be pushed off the culture dish by the expanding foci. However, in general, there did not appear to be a significant increase in the amount of floating debris within the growth media. The exceptions were invariably in older cultures (d16d12), which appeared generally less healthy.

From the results presented in this section it would appear that the normal cells do not significantly increase in density over time, even as the foci expand and take up more space on the culture dish. The transformed cells do not appear to cause an increase in the level of normal-cell apoptosis and the normal cells do not appear to be physically pushed off the plate by the expanding foci. Evidence from the focus morphology analysis would suggest that the transformed cells can, to some extent, grow over the normal cell monolayer, however, the fact that foci are compact in comparison to control colonies, would suggest that they are restricted in doing so. In order to measure the level of overgrowth, attempts were made to transfect the normal cells with the β-gal gene and then repeat the focus experiments with β-gal$^-$ transformed cells. However, the normal cells were unreceptive to the transfection protocol and only transient transfectants were obtained. Time constraints meant that alternative transfection regimes could not be explored.

The data would suggest that if the normal cells shuffle up, the increase in density is dissipated over the surface of the culture dish and the increase in space taken up by the foci appears to be insufficient to generate detectable increases in the density of the normal cells.

Figure 3.20. Rat2 apoptosis levels in focus formation assays

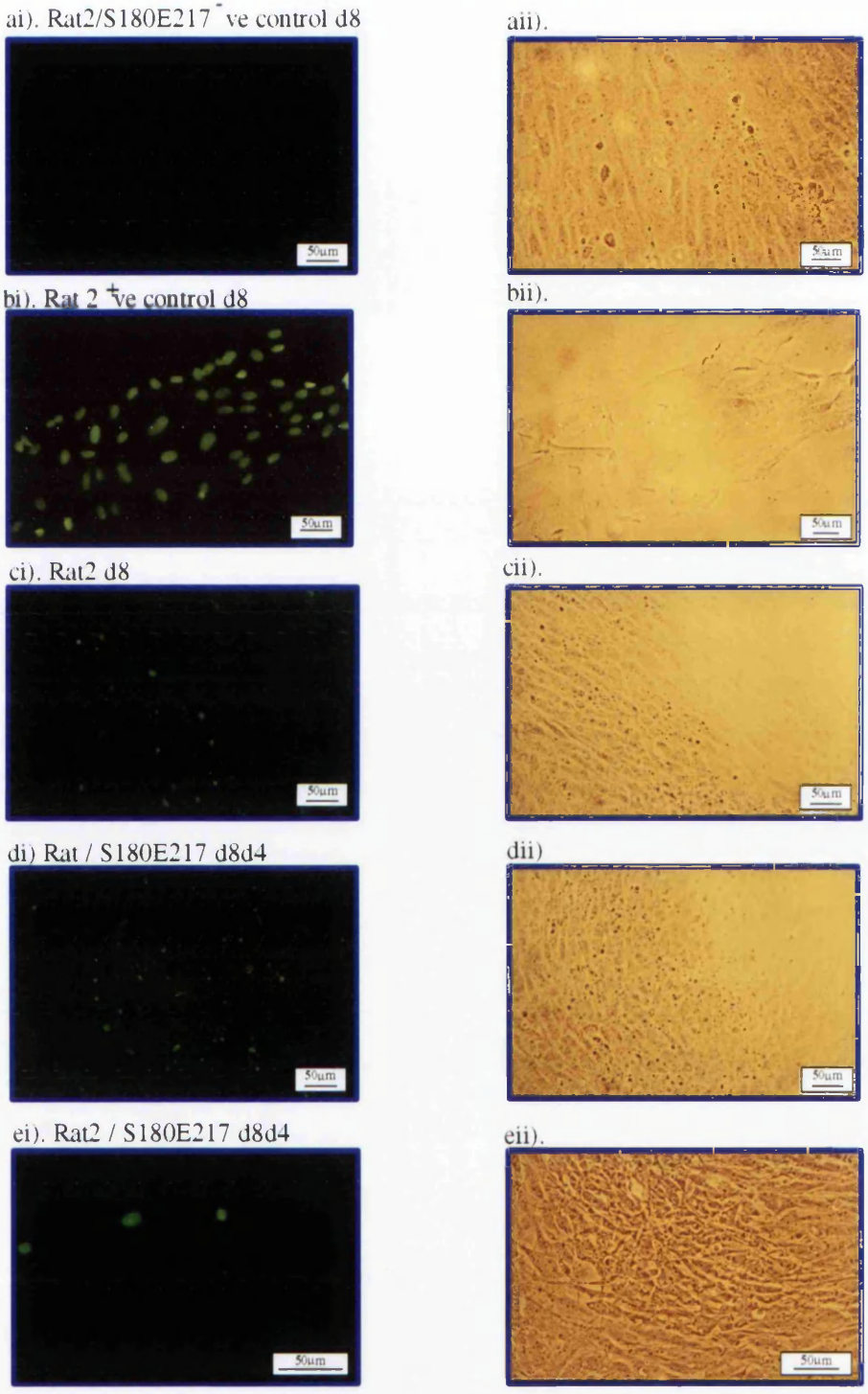


Figure 3.20. The above micrographs are examples from experiments designed to determine the level of apoptosis within the Rat2 monolayer when co-cultured with S180E217 cells. Rat2 and S180E217 cells were plated at 10^6 and 500 cells /dish respectively. Cell combinations and culture times are given above each micrograph. Figures ai) - ei) are immunofluorescence images of corresponding phase contrast images aii) - eii). Method of apoptosis detection is described in section 2.6. Positive and negative controls (Figures a & b respectively) were set up as described in section 2.6. The general level of apoptosis observed within a separate culture of Rat2 cells is shown in Figure c). Levels of apopotsis within co-cultures of Rat2/S180E217 cells was similar to controls (Figure d) and it can be seen in Figure e) that levels were no higher directly adjacent to S180E217 foci (Figure ei is at higher mag. than the corresponding phase contrast image -eii; no equivalent phase objective was available).

3.4.6.3. *Do transformed foci affect the proliferation rate of the normal cells?*

One advantage of the combined focus and proliferation assay developed for this project is that it provides direct evidence for the proliferation status of all cells in the culture dish. Observations made in earlier parts of the study would suggest that the growth of Rat2 cells close to transformed foci are growth stimulated. When investigating the inhibition phenomenon, Stoker also observed increased normal-cell proliferation. Stoker suggested that this was possibly due to the media being changed prior to analysis - an effect first observed by Todaro (1965). This possibility can be ruled out in the experiments carried out here because the culture media throughout the time course of the focus assays was not changed. Since Stokers initial observations there has been no further investigation into any possible growth stimulation phenomenon.

Growth stimulation of the normal cells adjacent to transformed foci may reduce the efficacy of the proposed inhibition mechanism since it has been shown that growth inhibition occurs only after the normal cells have reached saturation density (section 3.4.3) and are themselves growth inhibited. The proportion and distribution of stimulated normal cells has been analysed in more detail in this section.

At each time point, in the normal-cell control cultures, the average number of labelled and unlabelled cells per UA was determined from 20 UA's. The results are plotted as percentage labelled cells in (Figure 3.21. A & B). At each time point in the co-cultures the level of normal-cell growth stimulation was measured within 0.175mm of the focus periphery. 5 foci were analysed and around each focus the number of labelled and unlabelled cells / UA was determined from 10 UA's (or the max. possible when foci were small). The results from around the 5 foci were averaged and plotted as percentage labelled cells in the graphs presented in Figure 3.22. In addition the level of background cell proliferation away from the focus periphery, at all time points, was determined from 20 sample UA's taken as far away as possible from any foci and the results also plotted in the graphs presented in Figure 3.22.

In the control cultures, the labelling index of Rat2 and 10T1/2 cells remains relatively constant over all time points (Figure 3.21. A & B). In contrast, a relatively high level of cell proliferation was observed next to most foci in most focus assays (Figure 3.22; e.g. Figure 3.23.a).

In Rat2/S180 co-cultures the level of Rat2 cell proliferation away from the foci (Figure 3.22.A) remains at similar levels to control cultures (Figures 3.21.A). However, a significantly higher proportion of Rat2 cells within 1 UA of S180 foci, are dividing. The level of proliferation remains relatively constant over time.

The level of Rat2 cell proliferation surrounding S180E217 foci (Figure 3.22.B) is also significantly higher than in control cultures and away from any foci (e.g. Figure

3.23.e). Furthermore, it appears that cells in direct contact with the focus periphery were less likely to be dividing than cells 2-3 cells away (e.g. Figure 3.23.e & f).

Although there was stimulation of Rat2 cells surrounding S180NII foci (Figure 3.22.C), the level of stimulation was generally less than that recorded next to S180 & S180E217 foci. This would appear to correlate with the level of proliferation within the foci (i.e. transformed cells which are not growth inhibited stimulate a greater proportion of normal cells). However, cell proliferation within S180 foci decreases over time (section 3.4.3 Figure 3.14.A) but this does not correspond to a decrease in Rat2 cell stimulation next to these foci. The size of the foci may be of more significance, i.e. the larger the focus the greater the disturbance to the surrounding monolayer, which may disrupt the contact-inhibition mechanism or the more transformed cells there are the greater the stimulatory signal, if the signal is mediated by a concentration-dependent factor.

The proportion of Rat2 cells dividing next to BICR foci is higher than that away from the foci and in control cultures (Figure 3.22.D; e.g. Figure 3.23. a & b). The highest level of stimulation is seen when the two cell types are co-cultured for 4 days. In the majority of instances the pattern of stimulation is similar to that observed next to S180 cells & S180 cadherin transfectants i.e. the majority of dividing cells appear to be situated 2 or 3 cells away from the focus periphery (e.g. Figure 3.23.c). However, it can be seen from the data in Figure 3.22.D that the level of growth stimulation decreases over time. The level of BICR proliferation with the foci remains relatively constant over time (Figure 3.14.D) and the foci continue to grow (Figure 3.11.D). This does not support the hypothesis that focus size in some way affects the level of surrounding stimulation. However, it is possible that the BICR cells deplete local concentrations of specific growth factors required for the normal cells to divide.

The proportion of 10T1/2 cells growing next to the S180 foci is not significantly different from cell growth away from the foci (Figure 3.22.E; e.g. Figure 3.23.d). However, 10T1/2 cell proliferation over the monolayer in general, at d4d4, d6d4 & d8d4, is higher than in control cultures. This level of proliferation is not seen in later time points. Experiments in section 3.3.1 show that 10T1/2 cells, when plated at an initial density of 10^6, reach saturation density within 4 days. It is not clear why the mechanism responsible for inhibiting the growth of the 10T1/2 cells appears to be less effective at day 4 in the presence of the S180 cells, but would suggest that a concentration-dependent soluble factor is not involved since there are fewer S180 cells at these time points.

In instances where there is increased normal cell growth it is not clear how it is mediated. Experiments carried out in section 3.3.4 showed that transformed cell conditioned media was not capable of stimulating high density cultures of normal cells. The fact that the stimulation is localised around the foci suggests that stimulation may be

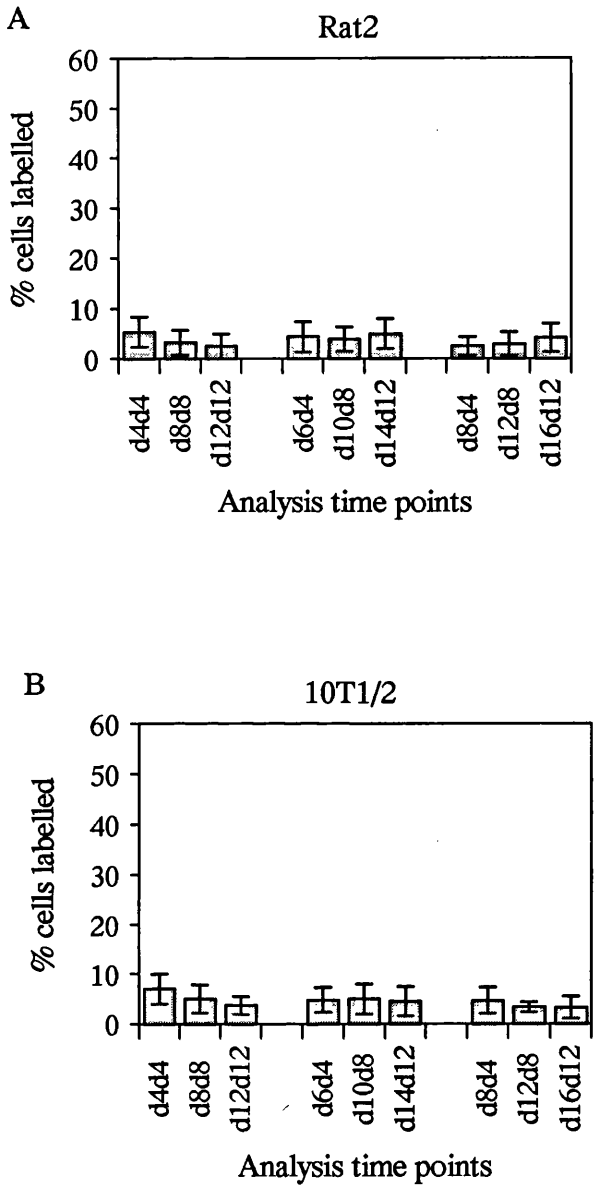


Figure 3.21. The level of proliferation within separate cultures of Rat2 and 10T1/2 cells.

Separate cultures of Rat2 and 10T1/2 cells were set up at 10^6 cells per dish. For each time point the number of dividing and non-dividing cells / UA was determined from 20 sample UA's. The % of dividing cells was calculated and the results presented above.

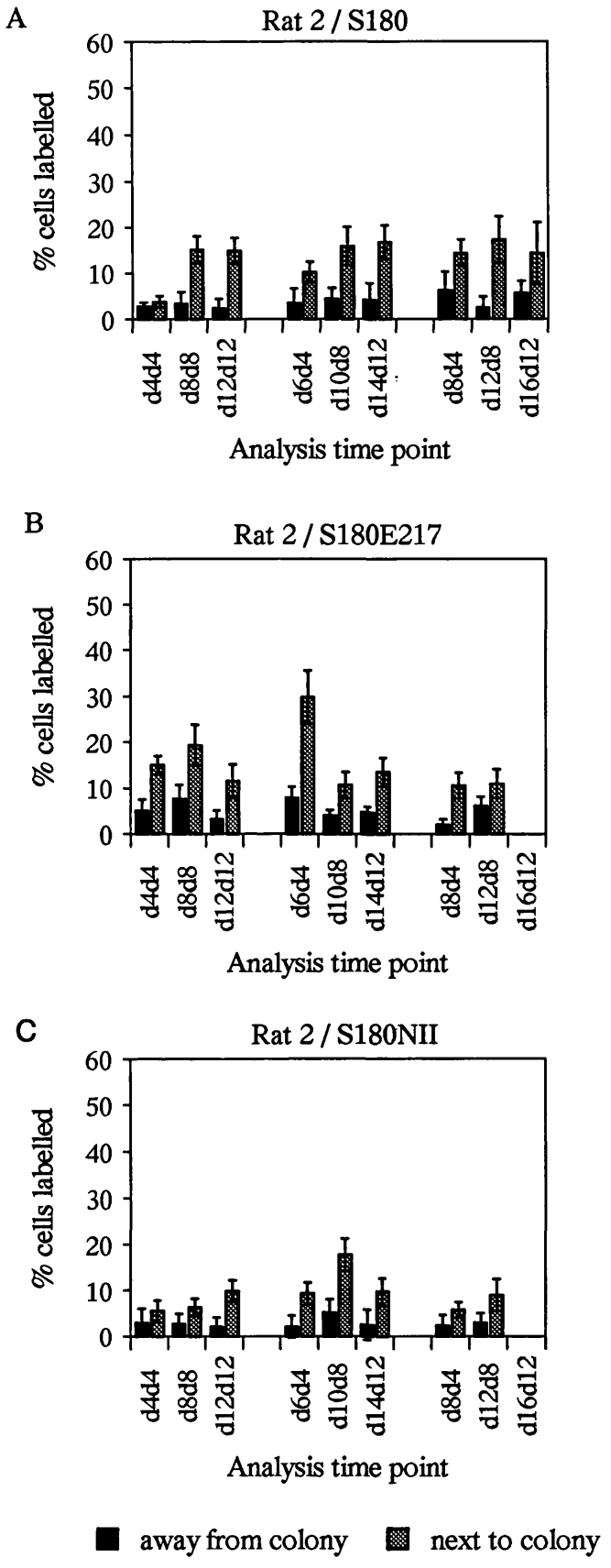


Figure 3.22. Labelling index of normal cells next to and away from focus periphery.

See Figure 3.15 for experimental details. At each time point 5 foci were examined. Around each focus the average number of dividing cells/UA within 0.175mm of the focus periphery was derived from 10 sample UA's. The results from around the 5 foci were averaged and presented as % labelled cells above. The % of labelled cells away from the focus periphery was determined after counting the number of labelled cells per UA in 20 sample UA's. No data was obtained at d16d12 in Rat2/S1870E217 & Rat2/S180NII co-cultures due to loss of cells during fixation.

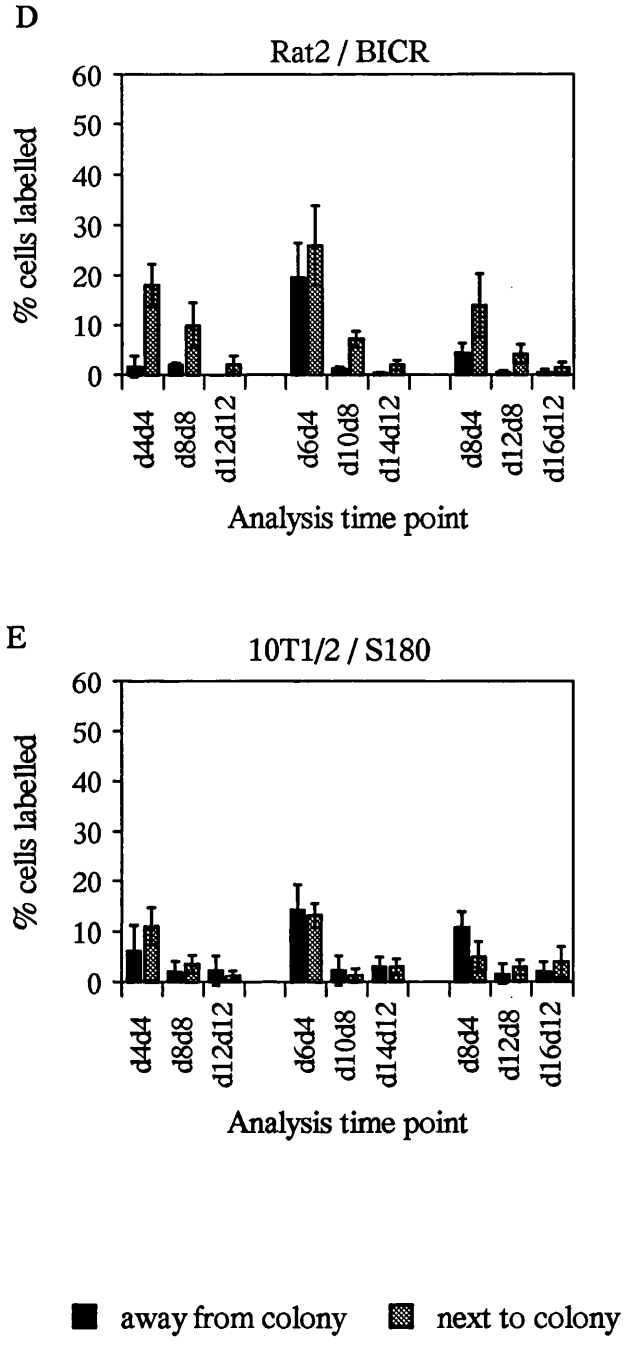


Figure 3.22. Continued: Labelling index of normal cells next to and away from focus periphery.

Figure 3.23. Stimulation of normal cells by transformed foci.

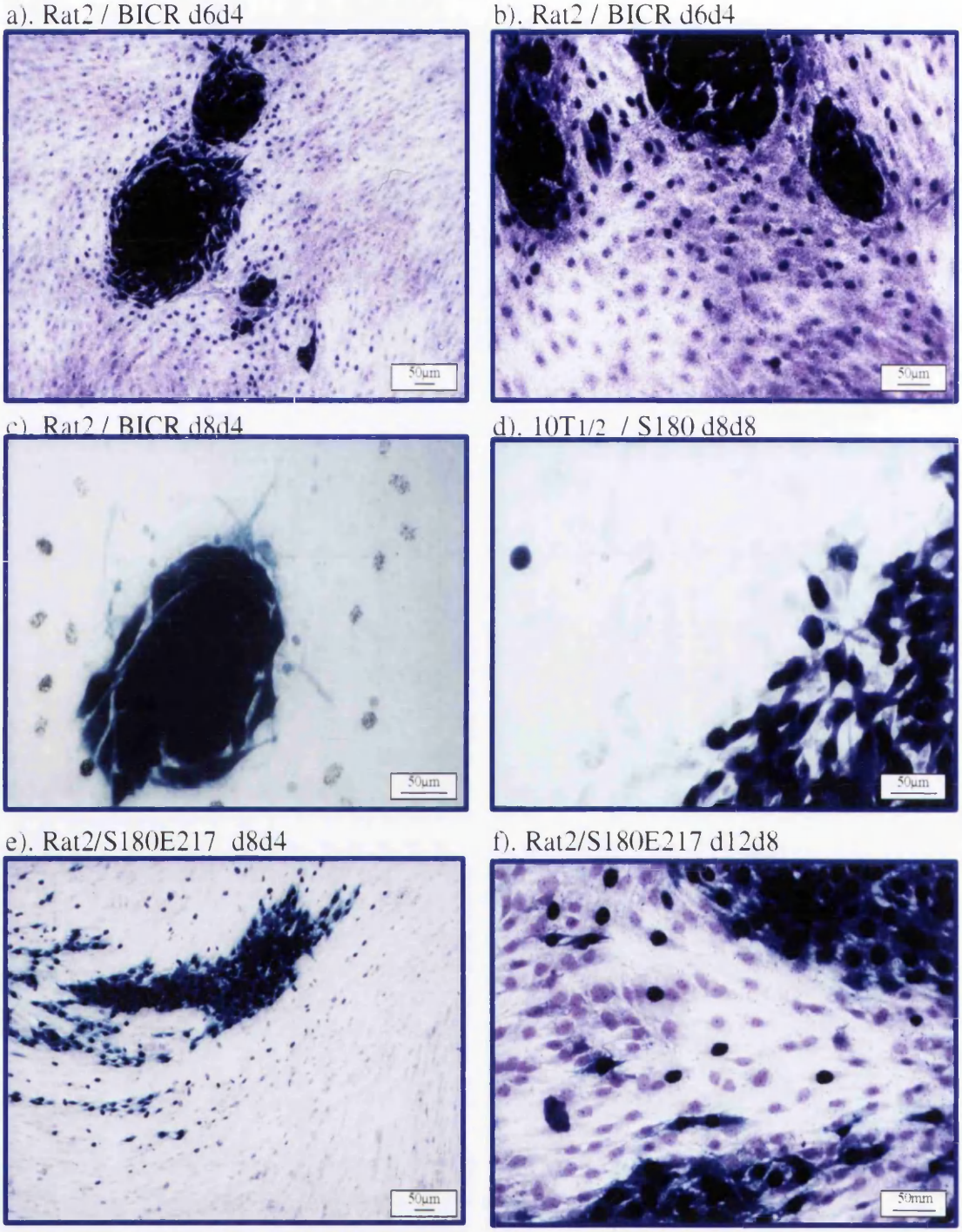


Figure 3.23. a)-f) show the effect of various transformed foci on the proliferative status of surrounding normal cells. The normal and transformed cells were plated at 10^6/ dish and 500/ dish respectively. The cell combinations and the culture times are given above each micrograph. Cultures were pulsed with [^{3}H]-thymidine (0.185MBq/ml) for 18hrs, then washed twice with PBS and fixed in 1% Formaldehyde and 0.1% Gluteraldehyde in PBS for 30mins. Cultures were then stained in x-gal for 8hrs, washed with PBS and processed for autoradiography (section 2.5). Figures a) & b) show increased levels of Rat2 cell growth localised around the BICR foci. In Figure c) this stimulation appears to be limited to a narrow band of cells surrounding the focus shown. In the majority of instances 10T1/2 cells were not growth stimulated by S180 foci, e.g. Figure d). In many instances growth stimulation appears to be more common when the Rat2 cells are close to but not in direct contact with the foci e.g. Figures c), e) & f).

caused by factors transmitted from the transformed cells. This may be achieved by a signal transmitted by GJIC or a paracrine factor (hence the localised effect). There is evidence to suggest that gap junctions mediate the stimulatory effect. This is on the basis that 10T1/2 and S180 cells do not appear to communicate with each other and 10T1/2 cells are not growth stimulated by the S180 foci. However, the level of Rat2 growth stimulation does not correlate with the level of communication with the the transformed cells. That is, the level of stimulation is comparitvely similar when the Rat2 cells are cultured with S180 cells (with which they are very poorly coupled) or with S180E217 cells & S180NII cells (with which they are well coupled).

Many of the Rat2 cells which are stimulated by the transformed foci are metabolically co-operating with the transformed cells via gap junctions. This is evident by the increased levels of [^{3}H]-thymidine labelling above individual, dividing Rat2 cells. For example, in Figure 3.26.a. a control population of Rat2 cells is shown with a relatively high proportion of cells dividing. Although the density of grains above the dividing cells is relatively low (Rat2 cells incorporate only low levels of [^{3}H]-thymidine due to a deficiency in thymidine kinase) it is sufficient to distinguish dividing cells from non-dividing cells. Rat2 cells dividing next to foci however, have a significantly higher grain density above their nuclei.

Growth stimulation may be caused by a physical disruption in the density-dependent growth inhibition mechanism of the normal cells and this is discussed further in section 3.4.6.4.

From these experiments it can be concluded that transformed foci appear to cause the growth stimulation of surrounding Rat2 cells but not 10T1/2 cells. However, the observations made during the analysis would suggest that cells in direct in contact with the foci are less likely to be dividing than those a few cells away. The pattern of Rat2 cell stimulation surrounding foci was examined in greater detail and the results presented in the following section.

3.4.6.4. *Is there a pattern to the stimulation of Rat2 cells surrounding foci?*

The pattern of Rat2 cell growth stimulation next to the transformed foci has been investigated. No analysis of the 10T1/2 / S180 co-culture was performed since, in the majority of instances, 10T1/2 cells showed no significant levels of growth stimulation next to the S180 foci. To analyse the pattern of stimulation the UA, measuring 0.175mm square, was subdivided into 10 rows, (Figure 3.24). The numbers of labelled cells in each row (designated by the distance from the focus periphery) were counted. 10 UA's were sampled around each focus (or max. possible when foci were small) and 5 foci were analysed for each time point; averaged results are presented in Figure 3.25. In instances where the periphery of the focus was uneven the transformed cells nearest to the normal cells were taken as being the focus edge.

Figure 3.24. Analysis of normal cell labelling pattern.

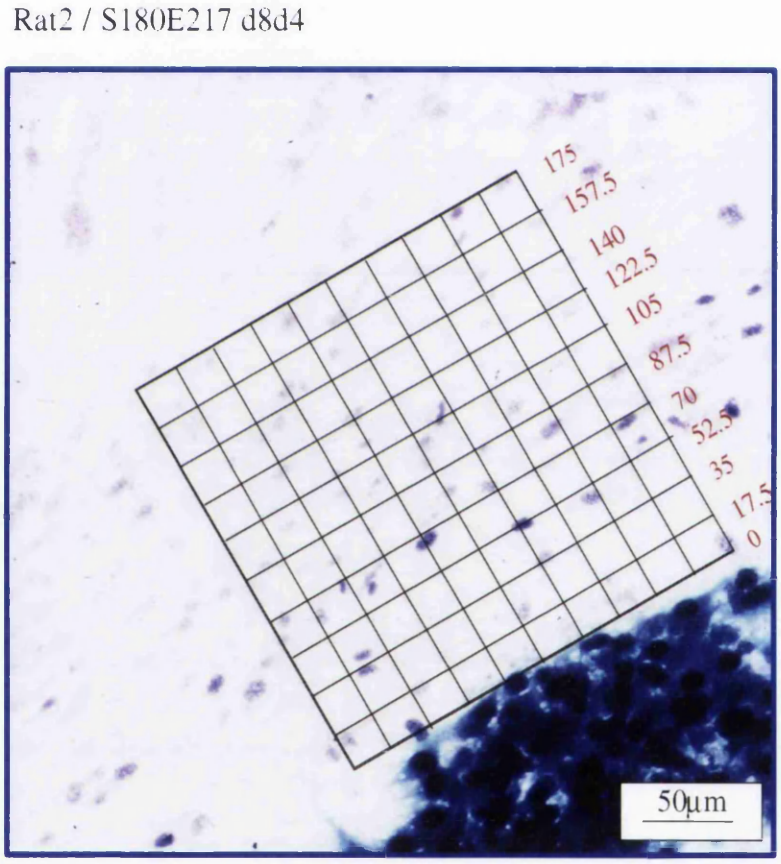


Figure 3.24. The above diagram shows a single UA placed at the edge of a focus. The number of labelled cells per row were counted. This was repeated 10 times around each of 5 foci per time point. The results were averaged and plotted in Figures 3.25.

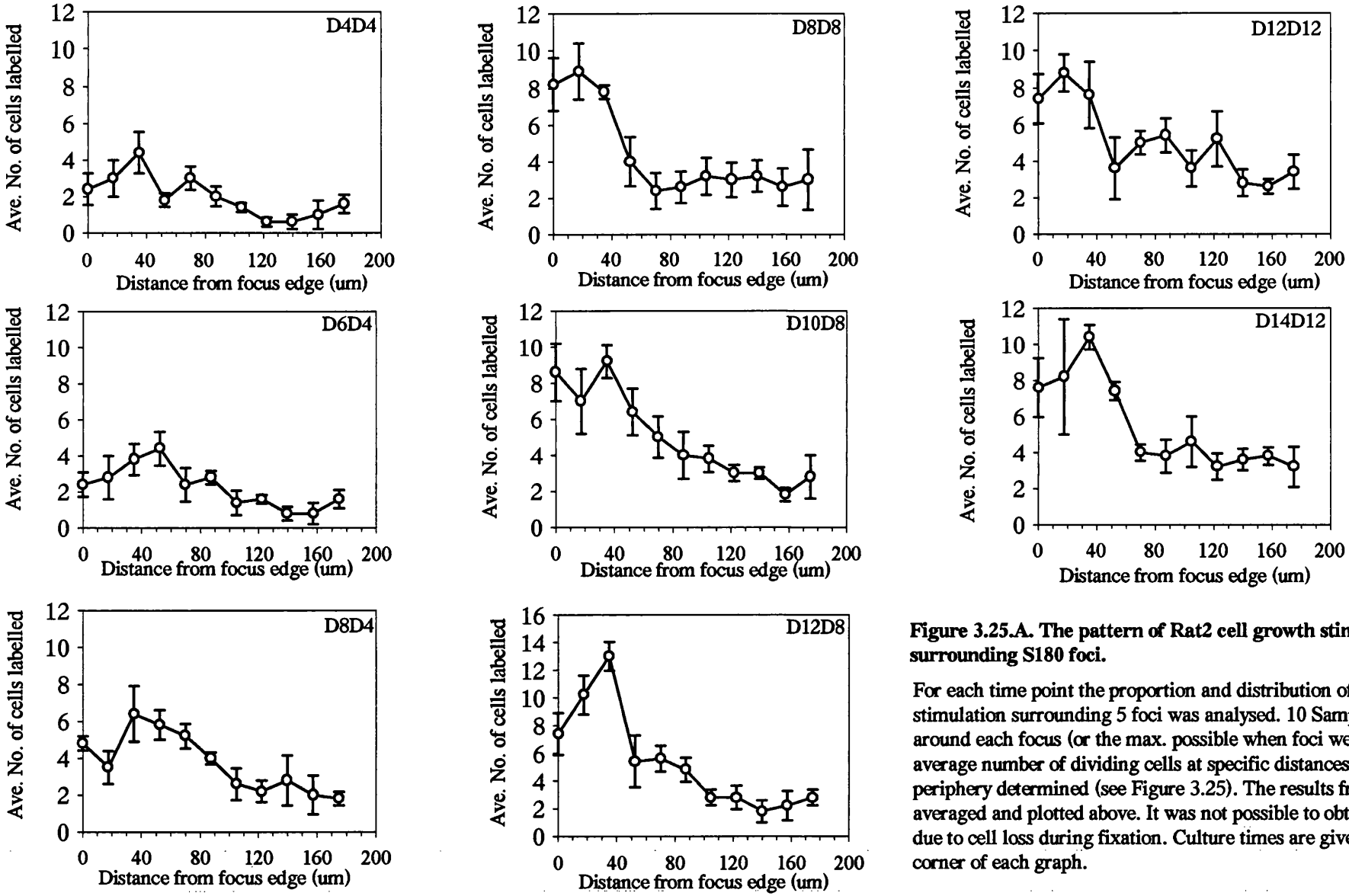
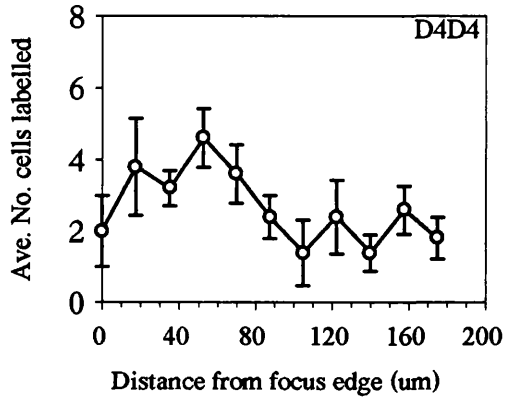
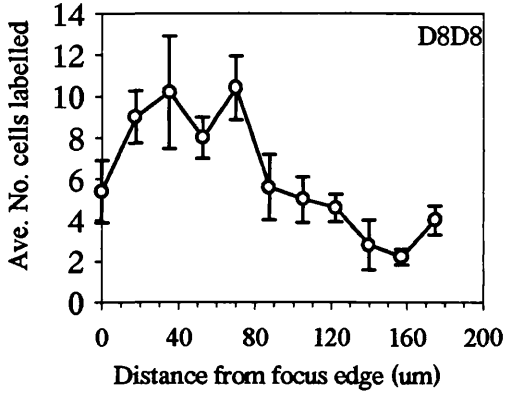
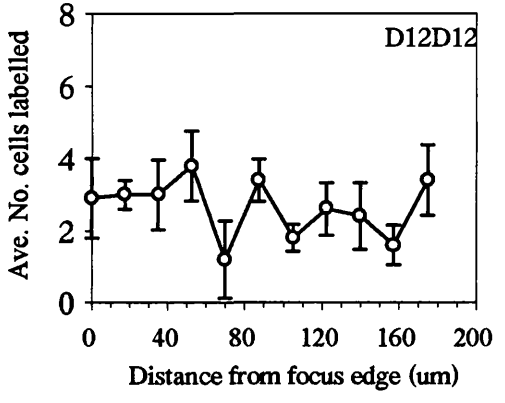


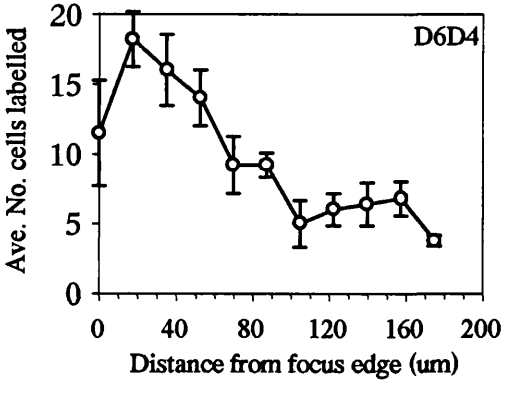
Figure 3.25.A. The pattern of Rat2 cell growth stimulation surrounding S180 foci.

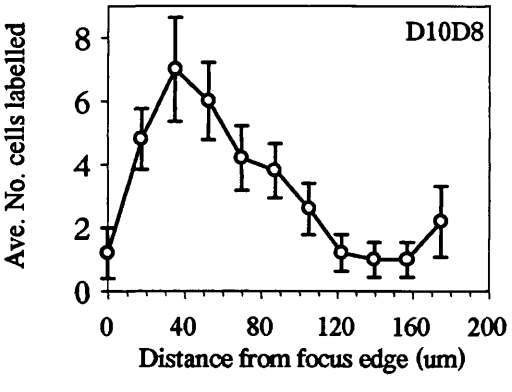
For each time point the proportion and distribution of Rat2 cell stimulation surrounding 5 foci was analysed. 10 Sample UA's were taken around each focus (or the max. possible when foci were small) and the average number of dividing cells at specific distances from the focus periphery determined (see Figure 3.25). The results from the 5 foci were averaged and plotted above. It was not possible to obtain data at D16D12 due to cell loss during fixation. Culture times are given in the top right corner of each graph.

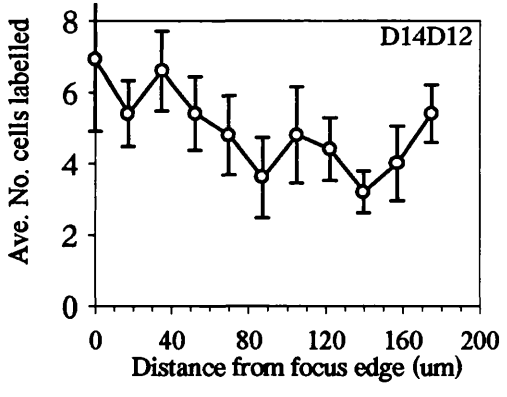


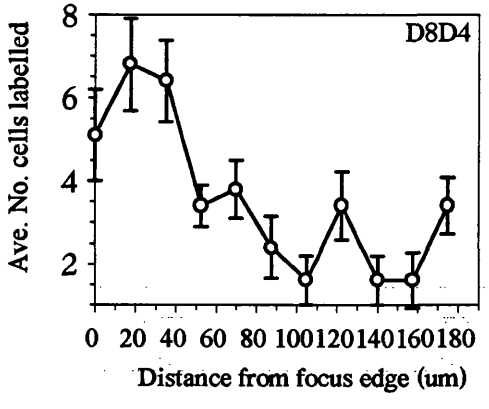












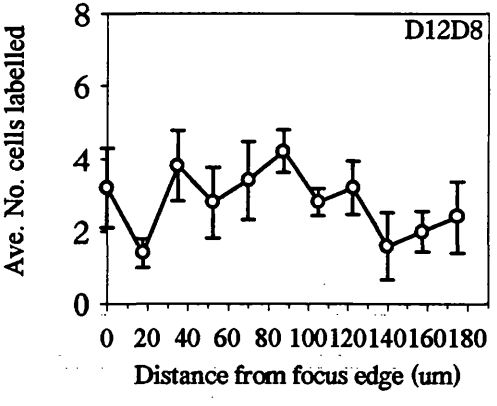
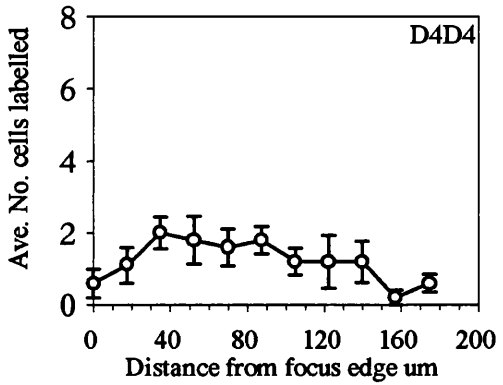
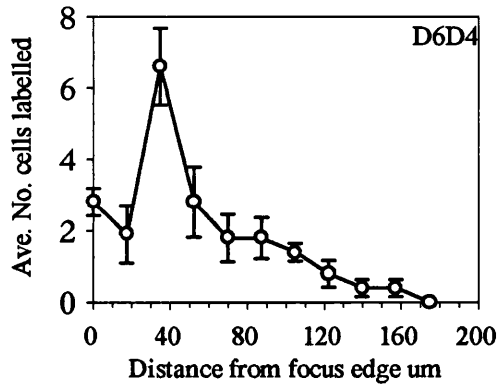
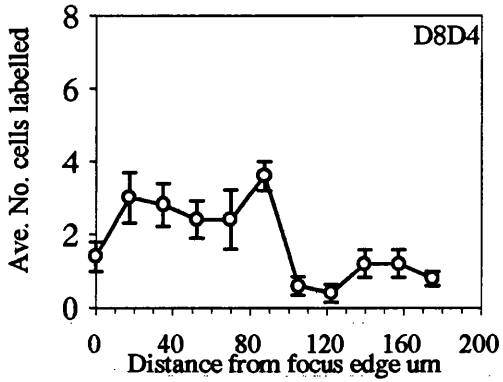


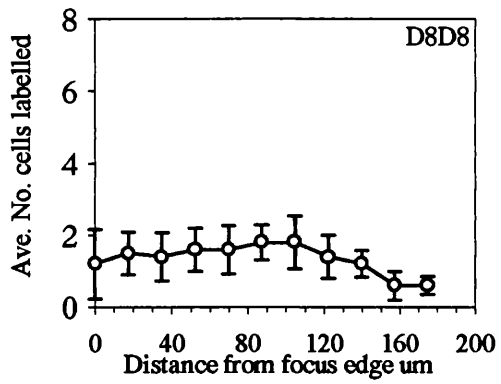
Figure 3.25.B. The pattern of Rat2 cell growth stimulation surrounding S180E217 foci.

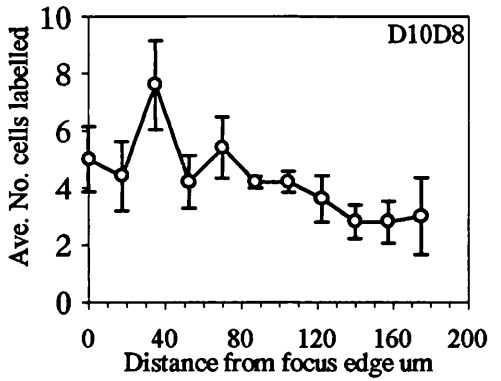
See Figure 3.26.A. for experimental details. Culture times are given in the top right corner of each graph. No data was obtained at D16D12 due to cell loss during fixation.

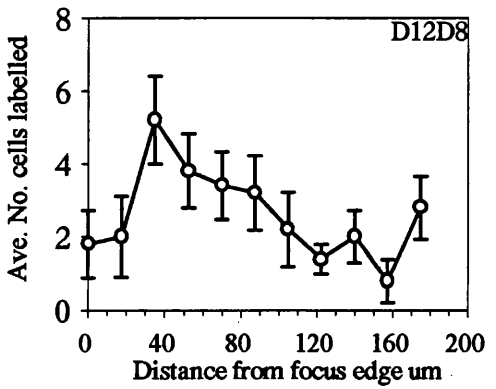


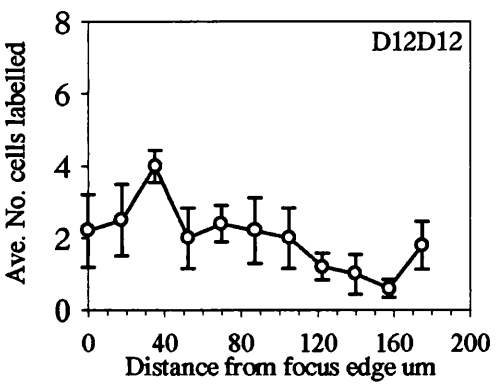












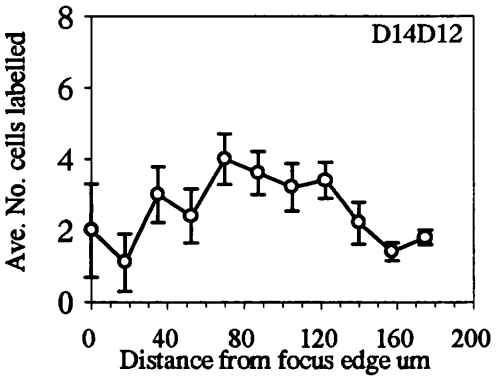


Figure 3.25.C. The pattern of Rat2 cell growth stimulation surrounding S180NII foci.

See Figure 3.26.A. for experimental details. Culture times are given in top right corner of each graph. No data was obtained at D16D12 due to cell loss during fixation.

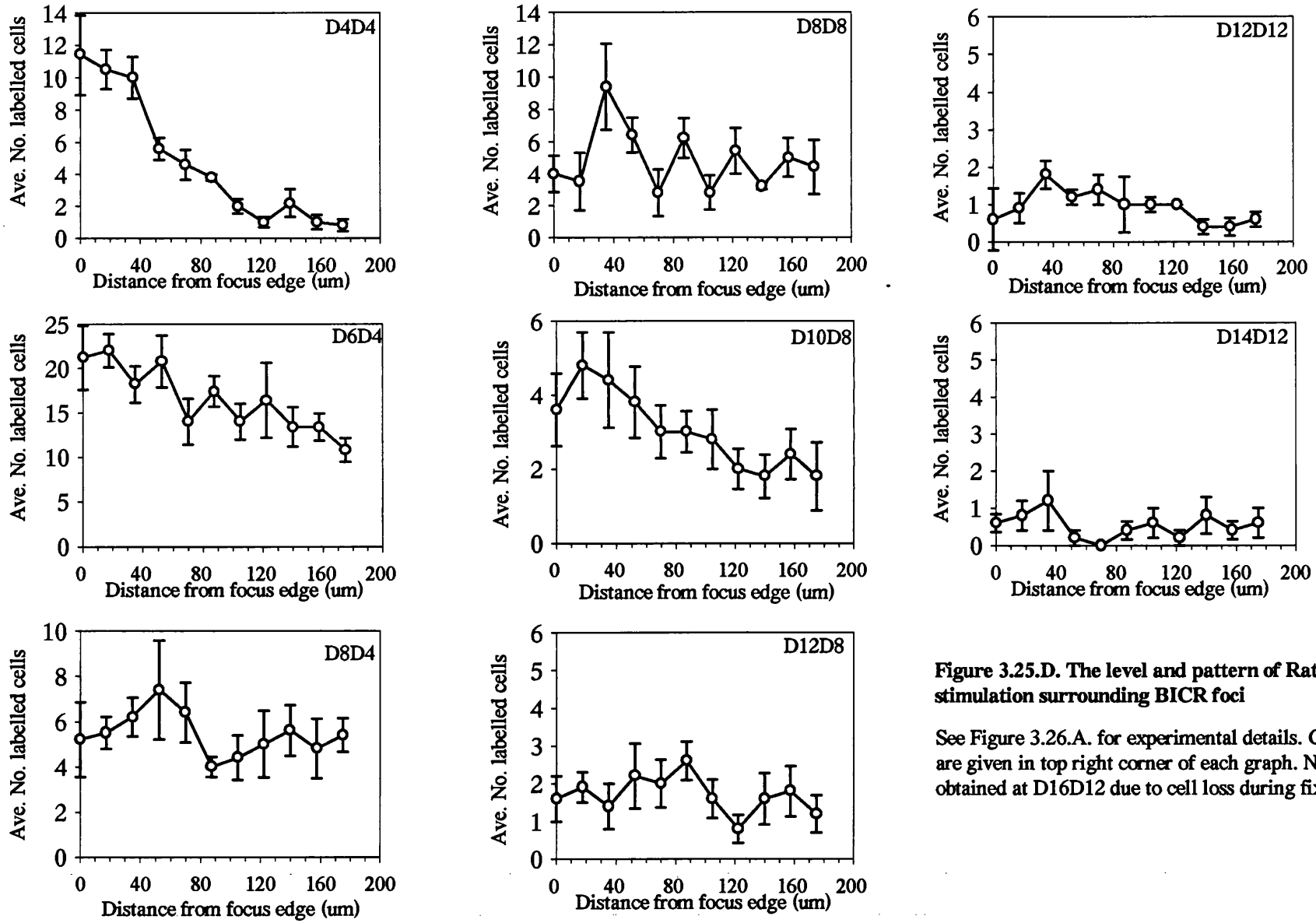


Figure 3.25.D. The level and pattern of Rat2 cell growth stimulation surrounding BICR foci

See Figure 3.26.A. for experimental details. Culture times are given in top right corner of each graph. No data was obtained at D16D12 due to cell loss during fixation.

Figure 3.26. Patterns of Rat2 cell division next to transformed foci.

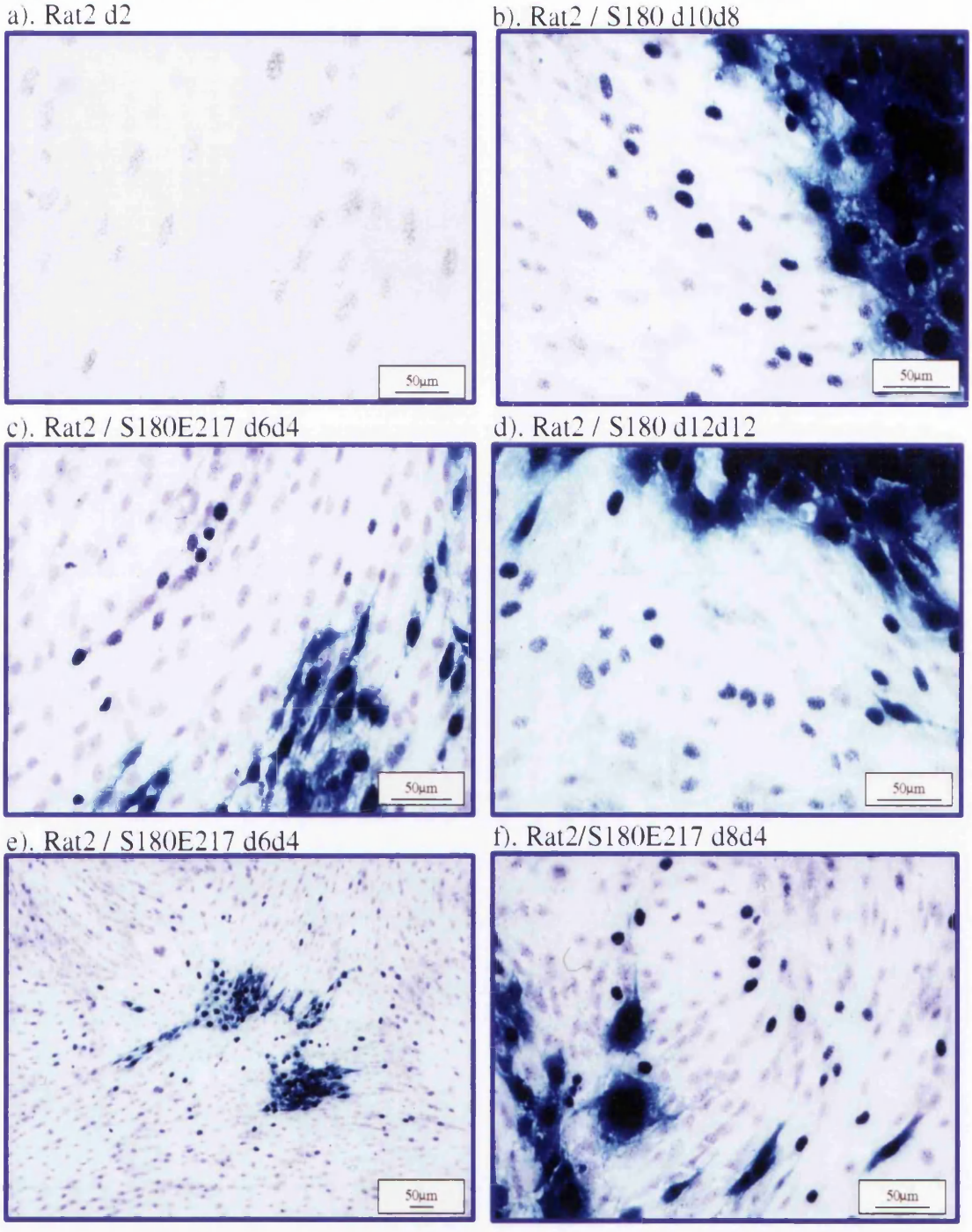


Figure 3.26. The above figures are examples of Rat2-cell growth stimulation next to various transformed foci. See Figure 3.24. for experimental details. Figure a) shows a control Rat2 culture ~2 days before reaching saturation density. Although the level of labelling is relatively low over individual cells (due to low thymidine kinase levels) it is sufficient to identify dividing cells. In Figure b) it can be seen that cells 30-50µm from the focus periphery are more likely to be labelled than cells in direct contact with the focus. Note also, the increased labelling over individual dividing Rat2 cells, this is indicative of metabolic co-operation. In Figures c) - f) examples are shown of the range of labelling patterns which were observed.

In all of the focus assays at the majority of time points (Figure 3.25. A - D), the Rat2 cells, near to the foci, are more likely to be dividing if they are situated 1 or 2 cells away (30-50μm) from the focus periphery rather than in direct contact with it. In all of the assays, the level of stimulation generally decreases with distance from the focus periphery. However, at the majority of time points, there appears to be a wave-like pattern of growth stimulation. Although the differences between the peaks and troughs of each wave is often only a few cells the pattern is consistently similar regardless of which transformed cell type forms the foci. Examples of different patterns of stimulation are shown in Figure 3.26.b, c, d, e & f. In many instances the labelled cells appeared to be in either groups or rows surrounding the focus.

Data from the previous section (3.4.6.1) would suggest that stimulation of normal cell growth surrounding the foci is not due to the 'removal' of Rat2 cells, thereby making room for other cells to gain space and divide. There is evidence to suggest that the stimulation of the normal cells is caused by a specific signal(s). This is based on the data which shows that the level of growth stimulation decreases with distance from the focus periphery and would suggest some form of concentration dependence on a factor is involved.

If such a signal is involved it may be transmitted via gap junctions. This is based on the observation that stimulation only occurs between cell combinations which show heterologous communication. However, this hypothesis and that of a single concentration-dependent paracrine factor is weakened by several lines of evidence. Firstly, the level of Rat2 stimulation by the transformed cells does not correlate with the level of communication between the two cells types. Furthermore, the pattern of stimulation recorded is not compatible with the junctional or paracrine hypothesis, i.e. normal cells which are in direct contact with the transformed foci are less likely to be labelled than cells 2-3 cells away. If the signal were to be passed via gap junctions or in a paracrine fashion then cells in direct contact with the transformed cells would receive the signal first when it is at its most concentrated.

The growth stimulation may not necessarily arise from a direct signalling event but may be the result of a physical disturbance in the density-dependent contact inhibition mechanism which is responsible for the autonomous growth control of the Rat2 cells. This growth inhibition occurs when the normal cells reach high cell density (section 1.6) and may be responsible for the phenomenon of transformed cell growth inhibition first observed by Stoker (1967). However, the mechanism of contact inhibition is not known. Several models have been proposed and all require direct cell-cell contact and high cell density.

If the growth stimulation is caused by a concentration-dependent factor, transmitted via gap junctions or by a paracrine mechanism then it might be expected that when the transformed cells out-number the normal cells the proportion of normal-cell

stimulation would be higher. To examine this, experiments have been performed in which the transformed cells are cultured in excess of the normal cells. The results are presented next.

3.4.7. The affect of excess transformed cells on colonies of normal cells.

It has been shown that the presence of various transformed foci stimulates the growth of surrounding Rat2 cells. The level of stimulation decreases as distance from the focus periphery increases and there appears to be a wave-like pattern to the growth stimulation. It is not clear however, if the stimulation is caused by a specific stimulatory signal, transmitted via gap junctions, secreted from the transformed cells in a paracrine fashion or is the result of physical disruption to the contact-inhibition between cells.

To examine this possibility, experiments were set-up in which the ratio of normal to transformed cells (usually 10^6 and 500 per dish respectively) was reversed. Thus any signalling factors transmitted from the transformed cells would pass into a relatively small pool of Rat2 cells and presumably reach high concentrations leading to a more pronounced effect.

Co-cultures of S180/Rat2, S180E217/Rat2, S180NII/Rat2 and BICR/Rat2 and were set-up. Transformed cells were seeded at 10^6 cells/90mm dish and normal cells at 500 cells/90mm dish (in triplicate). The two cell types were either plated simultaneously and analysed after 4 days co-culture or the normal cells were pre-established for 4 days the transformed cells then added and the co-cultures analysed after a further 4 days. As controls, separate cultures of normal and transformed cells seeded at 500 and 10^6 per dish respectively, were set-up in triplicate and analysed at the same time points as respective co-cultures. Cultures were pulsed with [^{3}H]-thymidine (0.185MBq/ml) for 18 hours prior to fixation in 1% formaldehyde and 0.1% gluteraldehyde in PBS for 30 mins. Cultures were then stained with x-gal and processed for autoradiography according to the method in section 2.5.

At each time point the average normal-cell colony area in control and co-cultures was determined after measuring 40 colonies (or max. possible if less than 40 formed). Average cell density (cells/UA) within 5 colonies was determined. For each colony the number of cells / UA in 10 sample UA's (or max. possible when colonies were small), was counted, this was repeated for each of the 5 colonies and the average calculated. The same procedure as above was followed when determining the number of labelled cells within the colonies. The average number of labelled transformed cells/UA away from the colony periphery was determined from 20 sample UA's. The average number of labelled transformed cells/UA around 5 colonies was calculated from 10 sample UA's (or max. possible when colonies were small) taken within 0.175mm of the colony periphery.

In co-cultures of S180/Rat2 cells, many Rat2 colonies detached during the 4 day co-culture or during the fixation process, which would suggest that cells were unhealthy or dying. Because of these problems no analysis could be performed on this cell combination.

Data from the experiments using co-cultures of S180NII / Rat2 cells is presented in Table 3.13. Control colonies of Rat2 cells increase in size and cell density over the two culture periods. Their LI remains very high at both of the time points analysed (d4d4:100% & d8d4:95%). Rat2 colonies in co-culture with excess S180NII cells are significantly smaller than control colonies and have a slightly higher cell density. The LI of the Rat2 cells within the co-culture colonies is markedly lower than in controls which would indicate that some form of growth inhibition, by the transformed cells, is taking place. However, there does not appear to be a pattern to the distribution of the labelled Rat2 cells within the colonies. The morphology of the colonies and the cells are not significantly different from control colonies and cells (e.g. Figure 3.27.a & b). Although the colony size data shows that colony expansion is restricted in the co-culture their morphology suggests that the transformed cells do not severely encroach on the colonies and compress the cells, unlike the normal cells in focus assays which compress and restrict expansion of the transformed foci (section 3.4.5). This would suggest that the Rat2 cells compete more effciently for space on the culture dish. The interface between the colonies and the surrounding transformed cell monolayer is distinct and there appears to be little or no overlap of either cell type which would suggest there is no overgrowth (e.g. Figure 3.27.b). Furthermore, the lack of increased density of S180NII cells around the Rat2 colonies, supports the possibility that the cells shuffle up when space on the culture dish is taken up by the expanding foci and would suggest that the S180NII cells have a lower efficiency for the plastic than the Rat2 cells.

The number of S180NII cells per UA in control cultures (d4d4:39, d8d4:46) is similar to that recorded in co-culture away from the colony periphery (d4d4:45, d8d4:48). There is no significant increase (P>5%) in the density of these cells in the presence of growing Rat2 colonies. The density of S180NII cells directly next to the colony periphery is not significantly higher than control or background levels (P>5%), e.g. Figure 3.27.b. and the labelling index of the S180NII cells remains constant regardless of whether the cells are situated next to or away from the colony periphery.

Table 3.13. The effect of excess S180NII cells on the growth of Rat2 cells.

Rat2 / S180NII	d4d4		d8d4	
	Control	Co-culture	Control	Co-culture
Ave. colony area mm^2	0.9 (0.4)	0.3 (0.1)	6.5 (0.3)	1.5 (0.1)
Ave. No. Rat2 cells per UA	6 (1)	10 (3)	21 (3)	17 (5)
Labelling index of Rat2 cells	100 (0)	20 (1.8)	95 (4.5)	11 (5)
Ave. No. S180NII cells/UA [away from colony edge]	39 (11)	45 (7)	46 (9)	48 (10)
Labelling index	45 (7)	47 (9)	36 (7)	51 (5)
Ave. No. S180NII cells /UA next to colony edge		42 (4)		38 (4)
Labelling index		38 (11)		40 (3)

Table 3.13. Controls were represented by separate cultures of Rat2 and S180NII cells seeded at 500 and 10^6 cells per 90mm dish respectively. In co-cultures the cells were seeded at the same respective densities as above. Rat2 cells were either seeded simultaneously with the S180NII cells or pre-established for 4 days at which point the transformed cells were added and co-cultured for 4 days. 18 hours prior to fixation (in 1% formaldehyde and 0.1% gluteraldehyde in PBS for 30mins), the cells were pulsed with [^{3}H]-thymidine (0.185MBq/ml). Once fixed, cultures were stained with x-gal and processed for autoradiography according to the method described in section 2.5. Standard deviations in parentheses.

Figure 3.27. Cell - cell interactions between excess transformed cells and normal cell colonies.

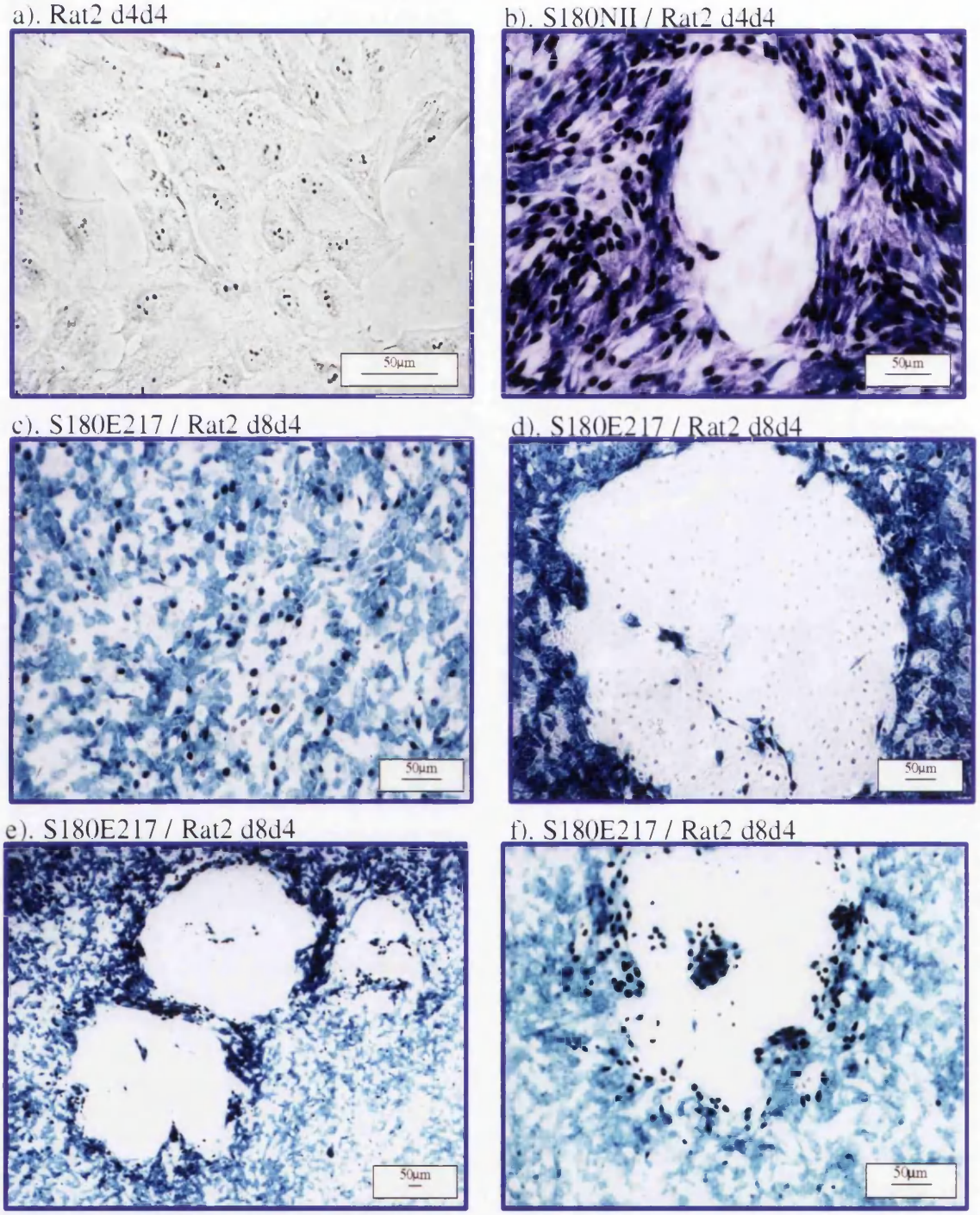
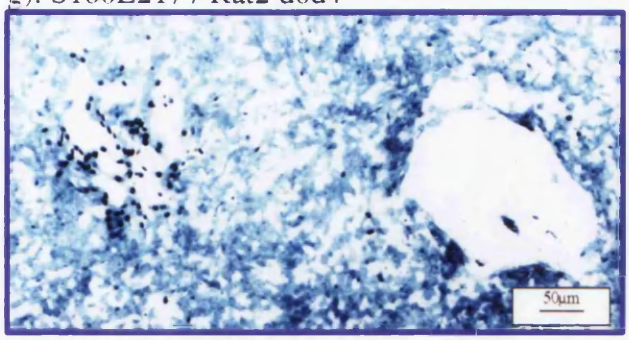


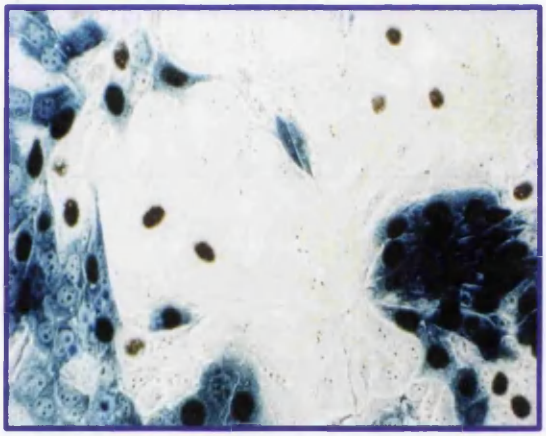
Figure 3.27. The above figures are examples from experiments performed to examine the effect of excess transformed cells on the growth of normal cells. Transformed and normal cells were seeded at 10^6 and 500 cells / 90mm dish. Controls were represented by separate cultures of each cell type seeded at the same densities as above. Cultures were pulsed with [^{3}H]-thymidine (0.185MBq/ml) for 18hrs, then washed twice in PBS and fixed in 1% Formaldehyde and 0.1% Gluteraldehyde in PBS for 30mins. Cultures were then stained in x-gal for 8hrs,washed with PBS and processed for autoradiography (section 2.5). Figure a) shows a Rat2 control colony. In Figure b) a Rat2 colony is surrounded by excess proliferating S180NII cells. Note that the Rat2 cells do not appear to be compressed. The level of S180E217 cell growth away from Rat2 colonies is shown in Figure c). In Figures d) - f) Rat2 colonies are surrounded by excess S180E217 cells. The Rat2 cells in d) do not appear to be compressed but do show relatively higher labelling than was observed in most Rat2 co-cultutre colonies. In Figures e) & f), S180E217 cell density and the proportion of cells dividing is greater next to the Rat2 colonies than away from them. Although a common observation, however, this effect was not observed around all Rat2 colonies e.g. Figure g) - see over page.

Figure 3.27. Cell-cell interactions between excess transformed cells and normal cell colonies.

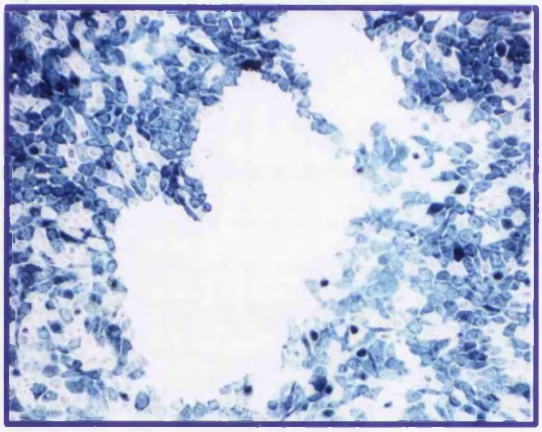
g). S180E217 / Rat2 d8d4



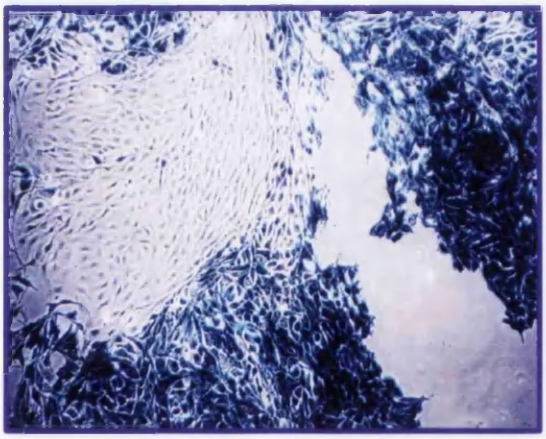
h). S180E217 / Rat2 d8d4



i). S180E217 / Rat2 d8d4



j). BICR / Rat2 d8d4



k). BICR / Rat2 d8d4

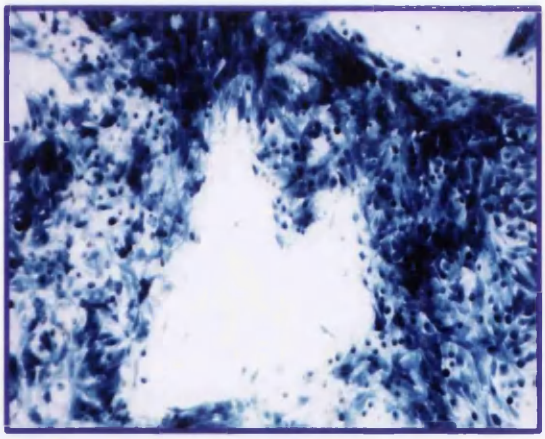


Figure 3.27:continued. The above micrographs are further examples from experiments designed to examine the effect of excess transformed cells on normal cell colonies. Figure g) illustrates the observation that S180E217 cells surrounding Rat2 colonies often, but not always, showed increased proliferation. In Figure h) it can be seen that the proportion of Rat2 cells dividing within the colony is relatively low and those which are, are generally not in direct contact with the transformed cells. Furthermore, the morphology of the Rat2 cells appears to be unaffected by the presence of the S180E217 cells. In Figure i) it can be seen that the Rat2 colony has completely detached from the culture dish, suggesting loss of cell-substrate adhesion. Many Rat2 colonies in co-culture with BICR cells also detached (e.g. Figure j). Increased density of BICR cells, but not proliferation, was also observed next to many Rat2 colonies (e.g. Figure k).

Data from the experiments using co-cultures of S180E217 / Rat2 cells is presented in Table 3.14. It can be seen from the data that Rat2 co-culture colonies are markedly smaller than control colonies. The cells are at a higher density per UA than in controls but their morphology would suggest that they are not severely compressed by the transformed cells (e.g. Figure 3.27.h) unlike the S180E217 foci formed in the presence of Rat2 cells (section 3.4.2) and would suggest that they compete better for space on the culture dish. In the majority of instances only a small proportion of Rat2 cells within the colonies are dividing (d4d4:24% and d8d4:17% compared to 100% - 95% in respective controls) and would suugest that the S180E217 cells are able to partially inhibit the growth of the Rat2 colonies. Even in the instances where Rat2 cell proliferation was relatively high such as that seen in Figure 3.27.d. it was not as high as controls.

The density and LI of the S180E217 cells away from the periphery of the colonies remains similar over both time points and in comparison to the controls (Table 3.14). However, an increase in S180E217 cell density is recorded around the periphery of Rat2 cell colonies and is particularly obvious at d8d4 (an increase of low significance, P=5% from 37 cells/UA to 48 cells/UA is recorded), for example, Figure 3.27.e. Furthermore, in many instances, the labelling index of S180E217 cells surrounding the Rat2 colonies is higher than the LI away from the colony periphery and in the control cultures (Table 3.14). This localised proliferation can be clearly seen in Figure 3.27.f and can be compared to background levels in Figure 3.27.c However, this labelling pattern was not observed around all colonies (~50% did not show increased labelling), e.g. Figure 3.27.g.

In several colonies examined at d8d4 it was apparent that the Rat2 cells had detached during the co-culture or fixing process e.g. Figure 3.27.i It can also be seen in Figure 3.27.i that β-gal expression is lost in a relatively high proportion of the S180E217 cells. This is probably because cells were grown in non-selective growth media during the co-culture period. Once cells were returned to selective media 100% β–gal expression was restored. Despite this problem it was possible to identify the two cell types in co-culture, firstly because Rat2 cells were present in distinct colonies and secondly there were morphological differences between the two cell types.

In many of the colonies the distribution of the labelled Rat2 cells was similar to the pattern observed next to transformed foci in section 3.4.6.3. That is, most labelled cells were situated 1-2 cells from the interface of the 2 cell types (e.g. Figure 3.27.h). However, this pattern was not observed in all colonies, so no statistical analysis was performed.

Table 3.14. The effect of excess S180E217 cells on the growth of Rat2 cells.

Rat2 / S180E217	d4d4		d8d4	
	Control	Co-culture	Control	Co-culture
Ave. colony area mm^2	0.9 (0.4)	0.2 (0.1)	6.5 (0.3)	0.8 (0.1)
Ave. No. Rat2 cells per UA.	6 (1)	11 (4)	21 (3)	50 (6.5)
Labelling index of Rat2 cells	100 (0)	24 (9)	95 (4.5)	17.6 (3)
Ave. No. S180E217 cells/UA [away from colony edge]	42 (9)	35 (5)	39 (3)	37 (2)
Labelling index	36 (7)	32 (12)	35 (3)	28 (4)
Ave. No. S180E217 cells next to colony edge		41 (5)		48 (2)
Labelling index		48 (8)		32 (4)

Table 3.14. Controls were represented by separate cultures of Rat2 and S180E217 cells seeded at 500 and 10^6 cells per 90mm dish respectively. In co-cultures the cells were seeded at the same respective densities as above. Rat2 cells were either seeded simultaneously with the S180E217 cells or pre-established for 4 days at which point the transformed cells were added and co-cultured for 4 days. See legend for Table 3.13 for experimental details. Standard deviations in parentheses.

The results obtained for BICR / Rat2 co-cultures are presented in Table 3.15. As with the other cell combinations examined the Rat2 colonies are markedly smaller than the control colonies. The majority of cells within the co-culture colonies are at a similar density to control colonies but have a considerably lower labelling index (Table 3.15). This would suggest that the presence of the BICR cells partially suppresses the growth of the Rat2 colonies. The loss of several colonies was observed in cultures analysed at d8d4 and an example of this can be found in Figure 3.27.j. The fact that all of the cells within these colonies (and in several Rat2 colonies in S180E217 co-cultures) detached as a single sheet of cells during the culture period would suggest that the cells have lost the ability to attach to the plastic rather than, or in addition to each other. The cells within the colonies which did remain (e.g. Figure 3.27.j) appear normal in terms of their general morphology and not markedly compressed by the presence of the surrounding BICR cells. In addition many of these cells are dividing which would suggest the cell detachment is not necessarily due to cell death.

The average density of BICR cells in the co-cultures remained approximately the same throughout the population. Although the average number of BICR cells at the colony periphery was not significantly different from the background and control densities there were occasional exceptions such as that seen in Figure 3.27.k. In most instances the extent of BICR cell division next to the Rat2 colonies was not significantly different to those away from the colony and unlike the Rat2 cells in some S180E217 co-cultures no specific labelling patterns were observed in the colonies themselves.

Table 3.15. The effect of excess BICR cells on the growth of Rat2 cells.

Rat2 / BICR	d4d4		d8d4	
	Control	Co-culture	Control	Co-culture
Ave. colony area mm^2	0.9 (0.4)	0.1 (0.1)	6.5 (0.3)	0.3 (0.1)
Ave. No. Rat2 cells per UA.	6 (1)	6 (1.5)	21 (3)	19 (3)
Labelling index of Rat2 cells	100 (0)	37 (12)	95 (4.5)	33 (4.8)
Ave. No. BICR cells/UA [away from colony edge]	37 (3)	42 (4)	40 (2.6)	40 (6)
Labelling index	24 (8)	55 (9)	31 (3.5)	45 (8)
Ave. No. BICR cells next to colony edge		45 (3)		43 (11)
Labelling index		43 (6)		37 (8.8)

Table 3.15. Controls were represented by separate cultures of Rat2 and BICR cells seeded at 500 and 10^6 cells per 90mm dish respectively. In co-cultures the cells were seeded at the same respective densities as above. Rat2 cells were either seeded simultaneously with the BICR cells or pre-established for 4 days at which point the transformed cells were added and co-cultured for 4 days. See legend for Table 3.13 for experimental details. Standard deviations in parentheses.

3.4.7.1. Summary.

The results presented in this section would suggest that the mechanism responsible for the stimulation of the Rat2 cells, observed in the focus assays, does not involve a specific concentration-dependent factor transmitted from the transformed cells. This is based on the observation that when a small population of Rat2 cells are surrounded by excess transformed cells, the proportion of Rat2 cells which are proliferating is relatively small. This is consistent with earlier suggestions that the distribution of dividing Rat2 cells surrounding the transformed foci was incompatible with a specific concentration-dependent factor.

In some instances the dividing Rat2 cells (surrounded by excess transformed cells) are situated towards the edge of the colony at the interface with the transformed cells (but not necessarily in direct contact with the transformed cells). This is similar to the distribution of dividing Rat2 cells seen in the focus assay experiments (stimulated Rat2 cells were situated with 2-3 cells from the focus periphery). These data would suggest that the growth stimulation arises through indirect interactions at the focus - monolayer interface. Further discussion of the possible mechanisms can be found in section 4.1.6.

In several instances when S180E217 cells were plated in excess of the Rat2 cells there was higher-than-background levels of S180E217 cell proliferation immediately next to the colony periphery. This stimulation may be caused by a specific signal arising from the Rat2 cells or alternatively, the normal cells may be contributing to their proliferation via some sort of feeder effect. However, the stimulation is unlikely to be mediated directly by gap junctions since the level of heterologous communication between Rat2 cells and S180NII cells (where there is not a significant increase in cell division surrounding the colonies) is higher than between Rat2 and S180E217 cells. The stimulation may be caused indirectly as a consequence of physical interaction between the two cell types; similar to that believed to be responsible for the stimulation Rat2 cells surrounding foci. It is not clear why this pattern occurred specifically with this cell combination and not with other transformed cell lines, particularly other S180 derived cells.

The results in section 3.4.3 show that the highest level of transformed-cell growth inhibition is observed with S180NII cells in co-culture with Rat2 cells. However, there appears to be no detectable inhibition of these cells when they surround the Rat2 colonies. This is consistent with the finding in section 3.4.3 that the larger the population of S180NII cells the lower the level of inhibition was imposed on them by the Rat2 cells. In addition there appears to be no growth stimulation of the S180NII cells surrounding the Rat2 colonies.

Rat2 cells within the colonies surrounded by transformed cells are generally at a higher cell density than control colonies, but show few signs of excess compression. This

is in contrast to the compressed transformed cells within foci surrounded by normal cells (section 3.4.5). This is consistent with the observations made in section 3.4.6. in which the morphology and density of the Rat2 cells surrounding expanding foci was very similar to control monolayers. This would appear to show that transformed cells do not encroach upon the normal cells and shuffle up as the Rat2 colonies expand.

Furthermore, the data would appear to show that Rat2 cells can compete better for space on the culture dish.

The data suggests that excess transformed cells are able to inhibit the growth of Rat2 cells. This inhibition phenomenon is not caused by nutrient deprivation and would appear to be caused by contact-inhibition or the ability of cells to respond to the presence of adjacent cells. This new inhibition phenomenon is discussed further in Chapter four in addition to possible mechanisms which may mediate the stimulation of Rat2 cells surrounding transformed foci.

CHAPTER FOUR

GENERAL DISCUSSION

4.1. Introduction.

Various normal and transformed cell lines, with a range of homologous and heterologous communication phenotypes, were selected to study the role of gap junctional communication in the inhibition of transformed cells by resting, excess, normal cells (see section 3.1 for cell lines). In the focus formation assays carried out here the majority of foci, in all of the co-cultures, were markedly smaller than respective control colonies (section 3.3). The difference in size was initially interpreted as growth suppression, however, further analysis revealed that cell density within many of the foci was significantly higher than cell density in respective control colonies. This would suggest that the physical presence of the normal cells restricts the transformed cells from gaining access to the culture dish, leading to increased focus cell density and results in a significant size difference between foci and colonies. In some instances cell density within the foci was too high to distinguish individual cells and it was therefore difficult to accurately identify the level of proliferation in these foci. However, the size of these foci together with the cell density data would suggest that inhibition was low. Two transformed cell lines were clearly shown to be growth inhibited; S180NII cells showed a high level of suppression when co-cultured with Rat2 cells and S180 cells showed a low level of suppression when co-cultured with 10T1/2 cells.

In some instances the difference in size between foci and respective control colonies was very large, even allowing for focus compression. For example, foci formed by S180E217 cells were composed of far fewer cells than respective control colonies. This would imply the cells are suppressed, however, a proliferation analysis showed that a high proportion of the cells were growing. The results suggest that a high proportion of S180E217 cells were detaching from the culture dish due to a lack of space, perhaps coupled with a defect in cell-substrate adhesion. Some foci formed by other transformed cells also showed large differences in size from respective control colonies. However, in most instances the difference was not as great as that seen with S180E217 foci and it is not clear to what extent the loss of cells from the dish contributed to the explanation of focus size suppression.

The data obtained in this study appears to contradict the generally held view that transfer of inhibition from normal to transformed cells, only involves GJIC. For example, transformed cell lines which communicate with the normal cells are not necessarily inhibited by them and the restoration of heterologous communication does not appear to be sufficient for inhibition to occur. Inhibition was also observed when heterologous communication was not detected (i.e. 10T1/2 / S180 co-cultures). Furthermore, the growth of normal cells was inhibited when they were co-cultured with excess transformed cells. This would appear to show that two different inhibition phenomenon exist, one which involves the transfer of inhibition from resting cells to growing cells

(possibly via gap junctions) and one which is, at least in part, due to contact-inhibition or the ability of cells to respond to the physical presence of other surrounding cells.

The results regarding the inhibition phenomena and the ability of the transformed cells to suppress the growth of normal cell colonies will be discussed in this section and the implications for the current working hypotheses considered. The limitations of the various experimental systems that are used to examine the phenomenon will also be discussed.

The advantage of the proliferation assay developed for this project is that it allows the growth of both cell types in the co-culture to be examined. During the proliferation analysis it was clear that Rat2 cells (but not 10T1/2 cells) surrounding the majority transformed foci were growth stimulated. The level of stimulation decreased as distance from the focus periphery increased and there appeared to be a wave-like pattern to the distribution of stimulated cells. It is not known what caused the stimulation but possible mechanisms are discussed.

4.1.2. Current hypotheses regarding the role junctional communication in the inhibition phenomenon.

It has been suggested that growth inhibition is transferred from normal cells to transformed cells via GJIC (Loewenstein 1979), however, the specific mechanism by which inhibition is imposed is not known; two possible mechanisms are frequently discussed. In one hypothesis the inhibition is mediated via a general homeostatic pressure set up within the coupled population of cells. Excess, cytoplasmic, stimulatory growth control molecules (e.g. second messengers), which are produced at higher levels by transformed cells, may be diluted by spreading to the normal cell monolayer. If this is so, a higher concentration of such factors may be found in normal cells surrounding the foci and may cause localised normal-cell growth stimulation. Limited evidence consistent with this has been obtained in this study and is discussed in section 4.1.6. Alternatively, the converse may occur. The contact-inhibition, in the normal cell monolayer, may be mediated by cell surface, membrane bound receptors that switch off the cell cycle. Interaction of the receptors on apposing membranes may trigger intracellular signalling cascades involving the release of second messengers that can diffuse through gap junctions into the transformed cells.

A second hypothesis, which is frequently discussed, suggests that specific growth inhibitory molecules, originating from discrete source cells within the normal cell population, pass through heterologous gap junctions into the transformed cells (Mehta et al 1986). The distribution of the signal is governed by junctional permeability and growth inhibition is therefore ruled by the number of channels and the extent of coupling. The level of inhibition will increase with the number of open channels and with the number of signal source cells. Working on the same principle it has been suggested (Mehta et al

1986 & 1991) that increased homologous communication within transformed cells can normalise their growth. However, no such inhibitory signalling molecule has been found and it is not clear why the production of such signals should be limited to random source cells.

In an attempt to show a role for gap junctions in the inhibition phenomenon, many investigators have used various chemical agents to modulate the level of communication between cells, in an attempt to show a correlation (section 1.3.4). However, these chemicals do not act exclusively on this pathway and other important cellular functions may be altered. In an attempt to provide direct evidence that GJIC is involved in the inhibition phenomenon connexins have also been used to manipulate the communication phenotype of transformed cells (Mehta et al 1991, Rose et al 1993, Chen et al 1995). The connexin transfectants often have a more normal phenotype in culture (e.g. longer PDT's, higher TD's and a flatter, more epithelioid morphology) and a more suppressible phenotype when co-cultured with normal cells. These changes are generally attributed to increased junctional communication and hence increased transfer of inhibition.

It is usually assumed that connexins are the channel components of gap junctions (which has yet to be proved; section 1.3.1), but evidence is accumulating to support the idea that they may have other functions. For example, a disease of the nervous system (X-linked Charcot-Marie-Tooth disease) which causes demyelination of axons is associated with mutations in the Cx32 gene (Bergoffen et al 1993). However, communication has not been shown to occur in mature Schwann cells and it is not clear what purpose intracellular gap junctions would serve, particularly considering the myelin sheath serves as an insulator. Cx32 may be involved in the formation of the myelin layers or contribute to the maintenance of myelin integrity (which is lost during the disease). This hypothesis is consistent with the idea that connexins are involved in bringing membranes into close contact in order that gap junctions can form (Finbow & Pitts 1993). The unusually close membrane apposition found in gap junctions is similar to that in myelin and such a role would explain the requirement of connexins for gap junction formation. This idea is supported further by the finding that connexins remain associated with dense cytoskeletal material when gap junctions (possibly formed by ductin) are extracted from mouse liver using Triton X-100 (Finbow & Meagher 1992). Connexins have also been implicated as tumour suppressor genes (Lee et al 1991) and regulators of gene expression. A recent paper by Chen et al (1995), showed that Cx43 expression can normalise the growth phenotype of transformed cells and alter their morphology (which is often associated with changes in the adhesion properties of the cells). In was not clear whether the changes in growth phenotype were due to the increased formation of gap junctions or due to changes in cell shape or expression levels of specific cell cycle regulatory genes including cyclin A, D1, D2 and the cyclin-dependent kinases 5 & 6.

4.1.3. The interpretation of focus formation data.

Much of the information regarding the inhibition phenomenon has been obtained from focus formation assays (e.g. Mehta et al 1986 & 1991, Martin et al 1991, Zhu et al 1992). Focus number and/or focus size are generally used as indices of growth. However, it has been shown in this study that values obtained from these measurements can vary considerably within an experiment due to some form of intrinsic variability, which is not understood (see section 3.3.6). Furthermore, the number of foci counted within an individual plate can vary depending on the size cut-off point which is used (section 3.3) and estimates of growth inhibition based only on focus size do not take into account the fact that foci can be compressed by the surrounding normal cells. Even in instances where inhibition is observed (e.g. 10T1/2 / S180 co-cultures) the level of transformed cell proliferation may be greater than the size of foci would suggest. Cell density within these foci can be markedly higher than in respective control colonies (e.g. Figure 3.12.E) and this would appear to contribute to the explanation of focus size suppression.

In some reports attempts have been made to calculate the proportion of transformed cells which have escaped inhibition by measuring the increase in total cell number within the co-culture over time (e.g. Mehta et al 1991). It is assumed that any increase in the number of cells within the co-culture is due to the growth of the transformed cell population (on the basis that separate cultures of normal cells remain static, in terms of total cell number, once they have reached saturation density). However, this does not take into account any increase in the number of normal cells due, for example, to growth stimulation by the transformed cells (see section 3.4.6.3). To overcome this problem the transformed cells could be transfected with the lineage marker β-gal and the cells of the co-culture counted using a FACS analysis (Fiering et al 1991). This technique would also overcome the problem, occasionally incurred in this study (section 3.4.2), of being unable to count the number of cells in each focus due to high focus cell density. However, the data obtained from such an analysis would not provide information on variability in growth within foci or between foci or whether focus size affects the extent of inhibition.

Although data from standard focus formation assays can be misinterpreted, some form of inhibition phenomenon has been shown to exist both here (section 3.4.3) and in several published reports (section 1.6). For example, Mehta and co-workers (1986) were able to show that foci formed from various transformed cells decreased in size as their level of heterologous communication with normal cells was increased by various chemical agents. In many instances the large differences in size between foci and colonies are unlikely to be due to focus compression alone. However, it has been shown in this study that foci which are markedly smaller than controls may not necessarily contain cells which are growth suppressed.

For example, an apparent discrepancy was frequently observed between the size of S180E217 foci and the proliferation status of the S180E217 cells (Figure 3.11.B & Figure 3.14.B). It is possible that S180E217 cells compete poorly for space on the culture dish which, coupled with high cell density within the culture, leads to the loss of cells from the focus to the medium and would therefore account for the reduced rate of focus expansion. The loss of cells is likely to occur whilst the cells are dividing, during which time they round up and the number of attachments to other cells and to the plastic may decrease. As the cells detach, surrounding cells may fill the space and divide. However, S180 parental cells did not appear to be affected in this way and high focus cell density would appear to contribute largely to the explanation of S180 focus size suppression. This would suggest that E cadherin expression in S180 cells decreases their ability to attach to the culture dish. It is not clear if N cadherin has the same effect on S180 cells because the S180NII cells were growth suppressed and did not reach a high cell density.

The data obtained from the combined focus and proliferation assay used in this study have shown the need to measure the growth status of the individual cells directly rather than trying to relate indirect growth indices to the level of proliferation within foci. It is possible that inhibition is greater when a single transformed cell is surrounded by growth arrested normal cells (a situation that would be analogous to the possible inhibition of a single mutated cell from which a tumour might develop). However, it has not been possible in this study to examine the phenotype of a single transformed cell within the monolayer of normal cells. This was because even when plated simultaneously with the normal cells the transformed cells went through 2 or 3 divisions before the normal cells had themselves stopped growing. One approach to overcome this problem would be to might be to seed the transformed cells on top of a confluent monolayer of normal cells which have already stopped dividing.

4.1.4. Do the data obtained in this study fit the current working hypothesis?

Mehta et al (1991) suggested that increased homologous communication can lead to the normalisation of transformed cells on the basis that gap junctions transmit growth-regulatory signals. It was found that MCA-10 cells (chemically transformed 10T1/2 cells) transfected with a cDNA for Cx43, show a 2-4 fold increase in homologous communication and have lower terminal densities and longer PDT's than parental controls. This hypothesis, however, would not appear to apply to the S180 series of cell lines examined here. Homologous communication in the S180 cells is markedly increased after they are transfected with a cDNA for E or N cadherin (Table 3.1). The cells become flatter with a decreased tendency to overlap. However, the PDT's and terminal densities of these cells is not significantly different from parental cells (Table 3.1). This may be because the transformed phenotype of the S180 cells is not directly associated

with the loss of cadherin expression or with the loss of communication, or the S180 cells may not produce the putative growth-regulatory control molecules. It is also possible that the results obtained by Mehta et al (see above) were not due to GJIC but to some other consequence of Cx43 expression, which may affect the growth control of cells via the regulation of specific cell cycle genes (see section 4.1.2). The S180 derived cell lines used here did not show an increase in Cx43 expression (but did show an increase in Cx43 phosphorylation; section 3.2.3).

In the introduction to the results (section 3.1) it was suggested that if GJIC was involved in the transfer of inhibition from the normal to the transformed cells it might be expected to see a greater level of inhibition at the periphery of large foci, where transformed cells receive the putative signal first and at the highest concentration, rather than in the centre. This did not appear to be the case. For example foci formed from pre-established S180NII cells showed a decrease in the level of suppression imposed by the Rat2 cells (relative to foci from cells plated at the same time; section 3.4.3) however, a significant proportion of cells were still suppressed but these cells were distributed, apparently evenly, throughout the focus and not with increased incidence at the interface with the surrounding monolayer.

It is often suggested that inhibition can occur even when the level of heterologous communication is very low. For example, Mehta et al (1991) found that MCA-10 cells are very poorly coupled to normal 10T1/2 cells. In focus formation assays these cells showed significant levels of inhibition (~37%; inhibition values are based on changes in total cell number). An MCA-10 Cx43 clone showed a 3-fold increase in heterologous communication with the 10T1/2 cells and the level of inhibition increased to ~99%. In this study S180 cells show a low level of communication with the Rat2 cells but do not appear to be suppressed (Figure 3.14.A). The S180E217 cells show a ~4-fold increase (relative to S180 cells) in their level of communication with the Rat2 cells but a very high proportion of S180E217 cells remain in cell cycle (Figure 3.14.B). S180NII cells which show a ~12-fold increase in heterologous communication do show a high degree of suppression (Figure 3.14.C).

It is not clear why N cadherin expression but not E cadherin expression can confer suppressible growth phenotypes on the S180 cells. Both cadherin transfectants show increased heterologous communication (relative to S180 parental cells) with the Rat2 cells, but appear to have different abilities to compete for space on the culture dish and this may be an influencing factor. These data would appear to show that GJIC is necessary but not sufficient for inhibition to occur and this is supported by the data which shows that BICR cells, which are well coupled to Rat2 cells, are poorly inhibited by them. There is also evidence from this study which shows that inhibition can occur in the absence of detectable GJIC, suggesting other factors may be involved.

In experiments where small numbers of Rat2 cells were plated with excess transformed cells, an unexpectedly high level of growth inhibition was recorded within 4

days in the Rat2 cell colonies, which would also suggest that other factors besides GJIC are involved in cell growth inhibition. It appears that growing transformed cells are able to suppress the growth of the Rat2 cells. It is unlikely that this inhibition is due to nutrient deprivation since media taken from cultures of an older age (8-10 days) can support normal Rat2 cell growth. These results suggest that the suppression of the transformed phenotype may be more complex than is often suggested. It is possible that other mechanisms may be involved in suppression and that GJIC is only a contributory factor.

4.1.5. Other mechanisms which may mediate growth inhibition.

The presence of junctional communication between the normal and transformed cells does not appear to be sufficient for inhibition to occur and other mechanisms may be involved. These are discussed next.

4.1.5.1. *Density-dependent regulation of cell growth (contact-inhibition).*

The growth of mammalian cells in tissues is thought to be under the control of various stimulatory and inhibitory factors. Inhibitory factors are presumably responsible for determining organ size and controlling tissue maintenance and may involve a specific inhibitory signal(s) which competes with the stimulatory signals or signals stimulation to be turned off. A considerable amount is now known about stimulatory stimuli such as mitogenic growth factors and their respective receptors. However, less is known about inhibitory factors and in particular the phenomenon known as *in vitro* density-dependent growth inhibition or contact-inhibition (section 1.6). This phenomenon describes the characteristic property of many mammalian cells to cease growing once they have reached high cell density within the culture dish (for normal cells this generally occurs soon after confluence is reached). The mechanism of contact-inhibition is not known but may be an important contributory factor to the Stoker phenomenon.

There are several lines of evidence, based on data obtained in this study, to suggest that the Stoker phenomenon does not only involve the direct transfer (via gap junctions) of inhibition from normal to transformed cells (section 4.1.4). The inhibition may be mediated by the ability of cells (normal or transformed, in separate or co-culture) to cease growing, or grow more slowly, at high cell densities. This may be determined by the ability of the cells to recognise the presence of adjacent cells and may be governed by their ability to compete for space on the culture dish. This in turn may be affected by how well the cells adhere to each other and to the plastic.

Evidence obtained throughout this study would suggest that the normal cells are better at competing for space than the transformed cells. For example, transformed foci show a large degree of compression and high cell density which would indicate that the cells are restricted in their ability to gain access to the plastic and some can survive more

easily on a smaller area. Furthermore, Rat2 cells show few signs of compression when they surround the foci (section 3.4.6) which would suggest they compete better for space and shuffle up in response to focus expansion. When low numbers of Rat2 cells are plated with excess transformed cells (section 3.4.7), the Rat2 colonies expand and do not show marked compression, supporting the suggestion that these cells are more effective at competing for space.

Cell proliferation analysis reveals that the normal cells (in standard focus assays) are contact-inhibited throughout the monolayer (section 3.4.6.3), although less so around transformed foci (this stimulation is addressed in section 4.1.6). When transformed cells are present in excess, the LI of the Rat2 cells within the colonies drops markedly in comparison to control colonies (section 3.4.7; e.g. Table 3.13). This shows that the transformed cells are able to inhibit the growth of the normal cells. This would suggest that a high proportion of Rat2 cells have competed with each other and with the transformed cells, which they recognise as an 'edge', for the amount of space they require to grow and divide, and as a consequence, become growth inhibited. In some instances (e.g. Figure 3.27.h, Table 3.14), Rat2 cells within the colonies which were dividing were found more frequently at the interface with the transformed cells. This would suggest that these cells are competing successfully for space with the transformed cells and can continue to divide.

An explanation of the observed growth inhibition in terms of the ability of the cells to compete for space on the culture dish seems consistent with much of the data, but not all. The inhibition of growth in S180NII foci by Rat2 cells, is the only instance of marked inhibition of transformed cell proliferation by normal cells in the focus assays (section 3.4.3). The cells in the inhibited foci are not significantly compressed and the cell density in the foci is often lower than in the control colonies. This suggests that these cells can effectively compete with the Rat2 cells for space. It would appear that they have sufficient space to divide but some other factor inhibits division (possibly mediated by GJIC). The inhibition is reduced when the foci are larger; this happens when the transformed cells are pre-established and the normal cells are added later (Figure 3.14.C). This suggests that inhibition is limited to the interface of the Rat2 and S180NII cells, but the inhibition is evenly distributed through the foci and not concentrated around the periphery. Conditioned media experiments show that the inhibition is not due to nutrient deprivation and is unlikely to be caused by secreted factors. These observations, unlike many others in this study (see section 4.1.4), are consistent with inhibition by transfer of small growth control molecules via gap junctions (either loss of stimulatory factors from the S180NII cells or invasion by inhibitory factors produced by normal cells). It appears that there are two separate phenomena which can cause growth suppression, one of which may require GJIC between the normal and transformed cells junctions and the other which can occur in the absence of heterologous communication and may depend on the ability of cells to recognise adjacent cells or an 'edge'.

4.1.5.2. *The role of cadherins and connexins in the inhibition phenomenon.*

Cadherins: Adhesion and communication events often go hand in hand, for example, increased adhesion between cells can lead to increased levels of homologous GJIC (Mege et al 1988, Keane et al 1988, Musil et al 1990, Jongen et al 1991, Meyer et al 1992, Hertig 1996) and it is therefore difficult to determine whether a particular change in cell behaviour is caused by one or the other. It has been suggested that cadherins may be involved in intercellular signalling and growth regulation by a pathway(s) not involving GJIC (see section 1.2.5.1). The effects of cadherins on growth are thought to be mediated by the cadherin-associated proteins, the catenins (Kinch et al 1995). β-catenin for example, has been shown to be phosphorylated in response to growth factors and can in turn decrease cell-cell adhesion. The E cadherin - catenin complex has been shown to retard the growth of a carcinoma cell line (Watabe et al 1994) and cadherin interactions between the normal and transformed cells may be involved in the inhibition observed in this study.

However, in the vast majority of instances, cadherin mediated adhesion requires homotypic interaction of the proteins on the apposing cell membranes; Rat2 cells do not express detectable levels of either N or E cadherin (section 3.2.3). However, adhesion between cells is a complex process and is the sum of contributory events (section 1.2.5). The expression of N cadherin (but, for some reason, not E cadherin) may alter the interactions between S180NII and Rat2 cells such that growth regulatory process are triggered when the two cell lines interact and may also affect the ability of the cells to compete for space.

Connexins. In several studies it has been shown that the expression of exogenous Cx43 in communication deficient transformed cell lines can restore junctional communication and a suppressible phenotype (see section 4.3.2). Chen et al (1995) showed that Cx43 transfectants were suppressed when co-cultured with normal cells. They also had altered expression levels of various proteins including cyclin A, D1, D2 and the cyclin dependent kinases, CDK5 and CDK6. Cx43 mutational analysis may provide the means to define specific connexin functional domains which may be responsible for these effects. At present, however, it is not possible to separate GJIC mediated events from other events which may be mediated by connexins. This may be overcome if specific inhibitors of junctional communication (aimed at ductin for example) become available which could be used to knockout junctional transfer but maintain connexin function.

In this study it was shown that E and N cadherin can increase the proportion of Cx43 which is phosphorylated. However, the increase in the expression of this Cx43 species does not appear to affect the growth parameters of these cell lines in homologous cultures (section 3.1). Furthermore, increased expression of phosphorylated Cx43 does not appear to affect the growth phenotype of the S180E217 cells in co-culture. BICR

and SVT2 cells also express relatively high levels of Cx43 (phosphorylated and unphosphorylated) but maintain aberrant growth phenotypes in homologous and heterologous co-cultures. If Cx43 is involved in the control of growth these data would suggest that the protein expressed in these cells is either defective or some other component is involved in the pathway which is not expressed or is not functioning in these cells.

It has been observed in several studies that connexin transfectants have a different morphology to parental cells; generally flatter with fewer processes (Mehta et al 1991, Rose et al 1993, Chen et al 1995). Changes in morphology are often associated with changes in cell-cell or cell-substrate adhesion (Marrs et al 1995, Gumbiner 1991) and would suggest that connexins are involved in or influence cell adhesion. In this study, the S180 cadherin transfectants had a different morphology to S180 parental cells. But is more likely that it is the cadherins which are causing the changes in cell morphology.

4.1.5.3. *Inhibition mediated by transmembrane bound glycoproteins.*

The molecular mechanism of contact-inhibition is not known but transmembrane glycoproteins have been implicated (Weiser 1990). It is suggested that these proteins interact in an adhesion-dependent manner with appropriate receptors on apposing cell membranes. A plasma membrane glycoprotein has been identified (contact-inhibin) that has density-dependent growth regulatory activity (Wieser 1990) when it binds to a specific 92kD protein (the contact-inhibin receptor; CiR) on apposing cells. Polyclonal antibodies against CiR release the cells from density-dependent growth control (Gradl et al 1995). SV40-transformed fibroblasts (different from the cells used in this study) were found to have reduced levels of CiR and a reduced capacity to bind to contact-inhibin. However, other transformed cells have been shown to express the protein in an active form but are not growth inhibited suggesting that the defects which led to their aberrant phenotype are located down stream of contact-inhibin. The role played by GJIC in these events was not studied.

It is not known if this form of growth inhibition has any significance for the results obtained here since there was insufficient time to analyse the cells for CiR and contact-inhibin expression. However, increased cell-cell adhesion mediated by cadherins or possibly by connexins may regulate the efficiency with which CiR and contact-inhibin bind to each other.

4.1.6. The stimulation of normal cells surrounding transformed foci.

Whilst investigating the inhibition phenomenon, Stoker (1967) observed that a higher proportion of normal cells (relative to controls) were dividing in the presence of the transformed cells. However, no detailed analysis was performed and it was suggested that the stimulation was due to the addition of fresh medium prior to the analysis. This

explanation could not account for the stimulation observed in this study (section 3.4.6.3) because the growth medium was not changed during the experiments. Since Stoker's original experiments, only the inhibition phenomenon has been examined in any detail and the possibility that transformed cells may alter the phenotype of surrounding normal cells has not been considered.

An increased proportion of Rat2 cells (but not 10T1/2 cells), surrounding the foci in most of the different co-cultures used in this study, was dividing (section 3.4.6.3). The stimulation was localised, generally within 0.175mm of the focus periphery and the proportion of dividing cells decreased as distance from the focus periphery increased. However, cells in direct contact with the focus periphery were less likely to be labelled than those 2-3 cells away and the distribution of dividing cells often followed a wave-like pattern (section 3.4.6.4).

The stimulation observed in this study is of interest in its own right but may also affect the ability of the normal cells to impose inhibition on the growth of the transformed cells. That is, by altering the surrounding environment to one which is not as growth inhibitory to the transformed cells, the level of suppression may be reduced. *In vivo,* at early stages of tumour formation if transformed cells were capable of stimulating the growth of surrounding normal cells they may have a selective growth advantage i.e. by reducing the ability of normal cells to impose growth inhibition the initiated cells could continue to divide, increasing the likelihood of more mutations which may provide the cell with further growth advantages.

4.1.6.1. *Possible mechanisms responsible for the stimulation of the normal cells.*

Several possible mechanisms may be involved in the stimulation of normal cells surrounding the transformed foci. The data from preliminary investigations carried out in this study do not provide conclusive evidence that any one in particular causes the stimulation but some would appear to be more likely candidates than others.

1. Stimulatory growth signals (e.g. paracrine growth factors) may be secreted from the cells and act in a localised and transient manner.
2. Stimulatory growth signals may be transmitted from the transformed cells to the normal cells via gap junctions.
3. The loss of normal cells from the culture dish as a consequence of competition for space with the transformed cells at the focus - monolayer interface may loosen up the monolayer or leave spaces which signal surrounding normal cells to divide.
4. The expanding foci may generate a physical disturbance which may disrupt the cell-cell contact-mediated inhibition which is responsible for the inhibiting the growth of normal cells at saturation density.

Paracrine factors: Conditioned media experiments would appear to show that stimulation is not mediated by a stable soluble factor secreted from the transformed cells. However, the factor may be short lived and require high concentrations which can only be achieved within a short distance from the source cell. In general, stimulation decreases with distance from the focus periphery suggesting a paracrine factor may be involved. However, more detailed analysis shows that the pattern of stimulation is more complex and is difficult to explain in terms of a single stimulatory factor. In experiments where low numbers of Rat2 cells are plated with excess transformed cells it might be expected that the level of stimulation would increase. However, the Rat2 cells show high levels of inhibition relative to controls and the interpretation of these experiments is more complicated. It appears unlikely that the stimulation is mediated by a paracrine factor secreted from the transformed cells.

Gap junction mediated stimulation: Some of the data are consistent with the idea that a growth stimulatory signal(s) is transmitted via gap junctions. In one cell combination (10T1/2 S180) there is a correlation between the absence of GJIC and the absence of stimulation (section 3.2.2.2; Table 3.3). It is also clear that Rat2 cells, which are stimulated near the transformed foci, are metabolically co-operating with the transformed cells. This is because there is an increase in the level of [^{3}H]-thymidine labelling above dividing Rat2 cells, relative to dividing Rat2 cells in separate cultures (see section 3.1 on the thymidine kinase deficiency in Rat2 cells). However, the level of heterologous communication does not correlate with the level of proliferation and the unexpectedly complex pattern of dividing Rat2 cells is not consistent with it being mediated by a single stimulatory factor transmitted by GJIC. To test whether gap junctions are involved in the stimulation mechanism, specific GJIC inhibitors would be required to block communication during the focus assay. These are not yet available.

Loss of cells from the dish: The loss of Rat2 cells surrounding the foci may, for a short time at least, leave a space on the dish which may allow other cells to fill the space and divide. However, it has been shown here (section 3.4.6.2) that the presence of transformed foci does not lead to increased normal-cell apoptosis. In experiments where the transformed cells were plated in excess of normal cells (section 3.4.7), the normal cell colonies which formed, continued to expand which would suggest they compete better for space than the transformed cells and are therefore unlikely to detach.

Disruption of the contact-inhibition mechanism: The stimulation may not be due to a specific stimulatory factor, but may be caused by the disruption of the contact-inhibition mechanism which causes the normal cells to cease growing at confluence and which may be responsible for some of the inhibition observed in Rat2 colonies surrounded by transformed cells. This disruption may be caused by expanding foci as the transformed

cells compete for space on the culture dish. As the transformed cells lay down new processes in between the Rat2 cells they may disrupt the surrounding monolayer such that the Rat2 cells are forced to shuffle up. As the foci continue to grow the Rat2 cells continue to shuffle back and it is possible that this may give rise to a wave-like pattern of stimulation. As distance from the focus periphery increases the disruption of the monolayer is dissipated and hence the number of cells dividing decreases.

It was shown in section 3.4.6.4 that Rat2 cells which were in direct contact with the transformed cells, were less likely to be stimulated than cells 2-3 cells away. Inhibition of Rat2 cells was observed when they were plated with excess transformed cells and this may have been caused by contact-inhibition. Therefore, in the focus assays, the Rat2 cells which are in direct contact with the transformed cells may remain contact-inhibited.

It is not clear why the 10T1/2 cells should not be stimulated when they are close to expanding S180 foci. The cell-cell contacts which lead to contact-inhibition may be stronger between the 10T1/2 cells and therefore reduce the disruptive effect of the transformed foci.

4.1.7. Summary

The inhibition of transformed cells by normal cells is more complex than is often suggested and more than one mechanism may be involved. It has been shown that the presence of excess transformed cells can inhibit the growth of normal cells. This is a new inhibition phenomenon which appears to be mediated by contact-inhibition and may be related to the ability of cells to compete for space on the culture dish. The frequency of inhibition reported in the literature may not be accurate. This is because indirect indices of growth, such as focus size, do not account for focus compaction by surrounding normal cells, which can contribute significantly to the explanation of focus size suppression. The assay developed for this study provides detailed and accurate information on cell growth and could be used to look for an inhibition phenomenon *in vivo*.

Adams J. C and Watt F. M. (1993). Regulation of development and differentiation by the extracellular matrix. *Development*. **117**. 1183-1198.

Akiyama S. K and Yamada K. M. (1993). Adhesion molecules in cancer. Part I. *Cancer Biology*. **4**. 215-218.

Alberts B, Bray D, Lewis J, Raff M, Roberts K and Watson J. D. (1989). *Molecular biology of the cell*. 2nd Ed. Garland Publishing Inc. New York.

Allen F., Tickle C and Warner A. (1990). The role of gap junctions in patterning of the chick limb bud. *Development*. **108**. 623-634.

Anklesaria P., Teixido J., Laiho M., Pierce J. H., Greenberger J and Massague J. (1990). Cell-cell adhesion mediated by binding of membrane-anchored transforming growth factor-α to epidermal growth factor receptors promotes cell proliferation. *Proc. Natl. Acad. Sci*. **87**. 3289-3293.

Apfel R, Lottspeich F, Hoppe J, Behl G and Bogdahn U. (1992). Purification and analysis of growth regulating proteins secreted by a human melanoma cell line. *Melanoma Res*. **2**. 327-336.

Armitage P and Doll R. (1954). *Br. J. Cancer*. **8**. 1-7.

Arteaga C. L, Tandon A. K, Hoff, D. D and Osborne C. K. (1988). Transforming growth factor β: potential autocrine growth inhibitor of oestrogen receptor-negative human breast cancer cells. *Cancer Res*. **48**, 3898-3904.

Asamoto M, Oyamada M, Aoumari A. E., Gros D and Yamasaki H. (1991). Molecular mechanisms of TPA-mediated inhibition of gap junctional intercellular communication: evidence for action on the assembly or function but not the expression of connexin 43 in rat liver epithelial cells. *Molecular Carcinogenesis*. **4**. 322-327.

Asashima M, Nakano H, Shimada K, Kinoshita K, Ishii K, Shibai H and Ueno N. (1990). Mesodermal induction in early amphibian embryos by activin A. *Devl. Biol.* **198**. 330-335

Atkinson M. M, Lampe P. D, Lin H. H, Kollander R, Li XR and Kiang D. T. (1995). Cyclic AMP modifes the cellular distribution of connexin43 and induces a persistent increase in the junctional permeability of mouse mammary tumour cells. *J. Cell Sci.* **108**. 3079-3090.

Atkinson M. M, Anderson S. K and Sheridan J. D. (1986). Modification of gap junctions in cells transformed by a temperature sensitive mutant of Rous sarcoma virus. *J. Membr. Biol*. **82**. 191-205.

Atkinson M. M, Menko A.S, Johnson R. G, Sheppard I. R and Sheridan J. (1981). Rapid and reversible reduction of junctional permeability in cells infected with a temperature sensitive mutant of avian sarcoma virus. *J. Cell Biol.* **9**. 573-578.

Atkinson M. M and Sheridan J. (1984). Decreased junctional permeability in cells transfected by different viral oncogenes: A quantitative video analysis. *J. Cell Biol.* **99.** 401a.

Azarnia R, Larsen W and Loewenstein W. R. (1974). The membrane junctions in communicating and non-communicating cells, their hybrids and segregants. *Proc. Natl. Acad. Sci.* **71.** 880-884.

Azarnia R and Loewenstein W. R. (1973). Parallel correction of cancerous growth and of a genetic defect of cell-cell comunication. *Nature*. **241.** 455-457.

Baker N. E, Mlodzic M and Rubin G. M. (1990). Spacing differentiation in the developing Drosophila eye: a fibrinogen-related lateral inhibitor encoded by scabrous. *Science*. **250.** 1370-1377.

Barrio L. C, Suchyna T, Bargiello T, Xu L. X, Roginski R. S, Bennett M. V. L and Nicholoson B. J. (1991). Gap junctions formed by connexins 26 and 32 alone and in combination are differently affected by voltage. *Proc. Natl. Acad. Sci.* **88.** 8410-8414.

Behrens J, Vakaet L, Friis R, Winterhager E, Roy F. R, Mareel M. M and Birchmeier W. (1993). Loss of epithelial differentiation and gain of invasiveness correlates with tyrosine phosphorylation of the E-cadherin / β-catenin complex in cells transformed with a temperature-sensitive v-src gene. *J. Cell. Biol.*. **120.** 757-766.

Bergoffen J, Scherer S. S, Wang M, Bone L. J, Paul D. L, Chen K, Lensch M. W, Chance P. F and Fishbeck K. H. (1993). Connexin mutations in X-linked Charcot-Marie-Tooth disease. *Science*. **262.** 2039-2042.

Berridge M. J. (1993). Inositol triphosphate and calcium signalling. *Nature*. **361.** 315-361.

Beyer E. C, Kistler J, Paul D. L and Goodenough D. A. (1989). Antisera directed against connexin43 peptides react with a 43kD protein localised to gap junctions in myocardium and other tisuues. *J. Cell. Biol.* **108.** 595-605.

Bi W. L, Parysek L. M, Warnick R and Stambrook P. J. (1993). *In vitro* evidence that metabolic co-operation is responsible for the bystander effect observed with HSV *tk* retroviral gene therapy. *Human Gene Therapy.* **4.** 725-731.

Bignami M, Rosa S, Falcone G, Tato F and Yamasaki H. (1988a). Specific viral oncogenes cause differential effects on cell-cell communication, relevant to the suppression of the transformed phenotype by normal cell. *Molecular Carcinogenesis*. **1.** 67-75.

Bignami M, Rosa S, La Rocca A, Falcone G and Tato F. (1988b). Differential influence of adjacent normal cells on the proliferation of mammalian cells transformed by the viral oncogenes *myc, ras* and *src*. *Oncogene*. **2.** 509-514.

Birchmeier W, Weidner M, Hulksen J and Behrens J. (1993). Molecular mechansims leading to cell junction (cadherin) deficiency in invasive carcinomas. *Cancer Biology*. **4.** 231-239.

Black F. M and Wakelam M. (1990). Desensitization of prostaglandin F2 alpha-stimulated inositol phosphate generation in NIH-3T3 fibroblasts transformed by normal c-Ha-ras-1, c-Ki-ras-1 and c-N-ras genes. *Biochem.* **267.** 809-813.

Bosenberg M. W and Massague J. (1993). Juxtacrine cell signalling molecules. *Current Biology.* **5.** 832-838.

Boucaut J. C, Darribere T, Boulekbache H and Thiery J. P. (1984). Prevention of gastrulation but not neurulation by anti bodies to fibronectin in amphibian embryos. *Nature.* **307.** 364-367.

Bradely R. S, Cowin P and Brown A. M. C. (1993). Expression of Wnt-1 in Pc12 cells results in modulation of plakoglobin and E-Cadherin and increased cellular adhesion. *J. Cell. Biol.* **123.** 1857-1865.

Bradley R. S and Brown A. M. (1995). A soluble form of Wnt-1 protein with mitogenic activity on mammary epithelial cells. *Mol. Cell. Biol.* **8.** 4616-4622.

Brauner T and Hulser D. F. (1990). Tumour cell invasion and gap junctional communication. *Invasion and Metastasis.* **10.** 31-48.

Brissette J. L, Kumar N. M, Gilula N. B, Hall J. E and Dotto G. P. (1994). Switch in gap junction protein expression is associated with selective changes in junctional permeability during keratinocyte differentiation. *Proc. Natl. Acad. Sci.* **91.** 6453-6457.

Brissette J. L, Kumar N. M, Gilula N. B and Dotto G. P. (1991). The tumour promoter 12-0-tetradecanoylphorbol-13-acetate and the ras oncogene modulate expression and phosphorylation of gap junction proteins. *Mol. Cell. Biol.* **11.** 5364-5371.

Brummer F. (1987). Farbstoffausbreitung durch gap junctions in tierischen zellulturen. PhD Thesis.

Bruzzone R, Haefliger J. A, Gimlich R. L and Paul D. L. (1993). Connexin 40, a component of gap junctions in vascular endothelium is restricted in its ability to interact with other connexins. Mol. Biol. *Cell.* **4.** 7-20.

Bruzzone R, White T. W and Paul D. L. (1994). Expression of chimeric connexins reveals new properties of the formation and gating behavior of gap junction channels. *J. Cell. Sci.* **107.** 955-967.

Budunova I. V, Carbajal S and Slaga T.J.(1995). The expression of gap junctional proteins during different stages of mouse skin carcinogenesis. *Carcinogenesis.* **16.** 2717-2724.

Buultjens T. E. J, Finbow M. E, Lane N. J and Pitts J. D. (1988). Tissue and species conservation of the vertebrate and arthropod forms of the low molecular weight (16-18000) gap junction proteins. *Cell Tiss. Res.* **251.** 571-580.

Buxton R.S and Magee A. I. (1992). Structure and interactions of desmosomal and other cadherins. *Seminars Cell Biol.* **3.** 157-167.

Carvalho A, Sanui H and Pace N. (1963). Calcium and magnesium binding properties of cell membrane materials. *J. Cell Comp. Physiol.* **62.** 311-315.

Caveney S. (1985). The role of gap junctions in development. *Ann. Rev. Physiol.* **47.** 319-335.

Chang C. C, Trosko J. Eambrosio G. D and Ambrosio M. D. (1987). Contact insensitivity of a subpopulation of normal human fetal kidney epithelial cells and of human carcinoma cell lines. *Cancer Res.* **47.** 1634-1645.

Chen Q, Kinch, M. S, Lin T. H, Burridge K and Juliano R. L. (1994). Integrin-mediated cell adhesion activates mitogen-activated protein kinases. *J. Biol. Chem.* **269.** 26602-26605.

Chen S. C, Pelletier D. B, Ao P and Boyton A. L. (1995). Connexin 43 reverses the phenotype of transformed cells and alters their expression of cyclin/cyclin dependent kinases. *Cell Growth & Differentiation.* **6.** 681-690.

Cho-Chung Y. S. (1985). The role of cAMP in the control of mammary tumour growth. In *Hormonally responsive tumours.* ed. Hollander V. P. pp 115-134. Academic press, Inc., New York.

Chow M, Yao A and Rubin H. (1994). Cellular epigenetics: topochronology of progressive "spontaneous" transformation of cells under growth restrtaint. *Proc. Natl. Acad. Sci.* **91.** 599-603.

Christian J. L, McMahon J. A and Moon R. T. (1991). Xwnt-8, a Xenopus Wnt-1/int-1 related gene responsive to mesoderm-inducing growth factors may play a role in ventral mesodermal patterning during embryogenesis. *Development.* **111.** 1045-1055.

Churchill D, Coodin S, Shivers R. R and Caveney S. (1993). Rapid de novo formation of gap junctions between insect hemocytes in vitro: a freeze-fracture, dye transfer and patch-clamp study. *J. Cell. Science.* **104.** 763-772.

Citi S. (1993). The molecular organization of tight junctions. *J. Cell. Biol.* **121.** 485-489.

Coffey R. J, Snipes, N. J, Bascom C. C, Weissman B. E and Moses H. L. (1988). Growth modulation of mouse keratinocytes by transforming growth factors. *Cancer Res.* **48.** 1596-1602.

Cohn M. J, Izpisua-Belmonte J. C, Abud H, Heath J. K and Tickle C. (1995). Fibroblast growth factors induce additional limb development from the flank of chick embryos. *Cell.* **80.** 739-746.

Connan G, Rassoulzadegan M and Cuzin F. (1985). Focus formation in rat fibroblasts exposed to a tumour promoter after transfer of polyoma *plt* and *myc* oncogenes. *Nature.* **314.** 277-279.

Cooke J. (1995). Morphogens in vertebrate development: how do they work? *Bioassays.* **17.** 93-96.

Cooper M and Pinkus H. (1977). Intrauterine transplantation of rat basal cell carcinoma: a model for reconversion of malignant to benign growth. *Cancer Res.* **37.** 2544-2452.

Crescenzi M, Crouch D. H and Tato F. (1994). Transformation by myc prevents fusion but not biochemical differentiation of c2c12 myoblasts: Mechanisms of phenotypic correction in mixed culture with normal cells. *J. Cell Biol.* **125**. 1137-1145.

Crow D. S, Beyer E.C, Paul D. L, Kobe S. S and Lau A. F. (1990). Phosphorylation of connexin 43 gap junction protein in unifected Rous sarcoma virus-transformed mammalian fibroblasts. *Mol. Cell. Biol.* **10**. 1754-1763.

Crowley C. W, Cohen R. L, Lucas B. K, Liu G, Shuman M. A and Levinson A. D. (1993). Prevention of metastasis by inhibition of the urokinase receptor. *Proc. Natl. Acad. Sci.* **90**. 5021-5025.

Culver K. W, Ram Z, Wallbridge S, Ishii H, Oldfield E. H and Blaese R. M. (1992). In vivo gene transfer with retroviral vector-producer cells for treatment of experimental brain tumours. *Science.* **256**. 1550-1552.

Dahl E, Winterhager E, Reub B, Traub O, Butterweck A and Willecke K. (1996). Expression of the gap junction proteins connexin31 and connexin43 correlates with communication compartments in extraembryonic tissues and in gastrulating mouse embryo respectively. *J. Cell Sci.* **109**. 191-197.

Dahl G, Miller T, Paul D. L, Voellmy R and Werner R. (1987). Expression of functional cell-cell channels from cloned rat liver gap junction cDNA. *Science*. **236**. 1290-1293.

Damsky C.H and Werb Z. (1992). Signal transduction by integrin receptors for extracellular matrix: cooperative processing of extracellular information. *Current Biology*. **4**. 772-781.

Davis R. J. (1993). The mitogen-activated protein kinase signal transduction pathway. *J. Biol. Chem.* **268**. 14553-14556.

De Laat S. W, Tertoolen L. G. J. Dorresteijn A. W. C. and Biggelar J. A. M. (1980). Intercellular communication patterns involved in cell determination in early molluscan development. *Nature*. **287**. 546-549.

DeHann R. L. (1988). Dynamic behavoir of cardiac gap junctionsa. in *Gap Junctions*. ed. Hertzberg E.L and Johnson R. G. pp 305-320. Alan Liss Inc., New York.

Demetriou M, Nabi I. R, Coppolino M, Dedhar S and Dennis J. W. (1995). Reduced contact inhibition and substratum adhesion in epithelial cells expressing GlcNAc-transferase-V. *J. Cell. Biol.* **130**. 383-392.

Dermietzel R, Traub O, Hwang E, Beyer M. V, Bennett D, Spary D. C, Willecke K. (1989). Differential expression of 3 gap junction proteins in developing and mature brain. Proc. Natl. Acad. Sci. 10148-10152.

Dermietzel R and Spray D. C. (1993). Gap junctions in the brain: where, what type, how many and why? *Trends Neurosci.* **5**. 186-192.

DeVries S. H and Schwartz E. A. (1989). Modulation of an electrical synapse between solitary pairs of catfish horizontal cells by dopamine and second messengers. *J. Physiol.* **414**. 351-375.

Dhawan J, Lighter A. C, Rowe D. W and Farmer S. R, (1991). Cell adhesion regulates pro-alpha 1 collagen mRNA stability and transcription in mouse fibroblasts. *J. Biol. Chem.* **266**. 8470-8475.

Diener B, Becker R, Martus H. J, Traiser M, Steinberg P and Oesch F. (1995). Malignantly transformed non-parenchymal liver epithelial cells and transformed oval cells suppress the homotypical gap junction intercellular communication of co-cultured rat liver parenchymal cells. *Carcinogenesis*. **16**. 633-636.

Dorudi S and Hart I. R. (1993). Mechanisms underlying invasion and metastasis. *Current Biol.* **5**. 130-135

Dotto G. P, Weinberg R. A and Ariza A. (1988). Malignant transformation of mouse primary keratinocytes by Harvey sarcoma virus and its modulation by surrounding normal cells. *Proc. Natl. Acad. Sci*. **85**. 6389-6393.

Duband J, Dufour S, Hatta K, Takeichi M, Edelman G. M and Theiry J. P. (1987). Adhesion molecules during somitogenesis in the avian embryo. *J. Cell. Biol.*. **104**. 1361-1374.

Duband J. L, Tucker G. C, Poole T. J, Vincent M, Aoyama H and Thiery J. P. (1985). How do the migratory and adhesive properties of the neural crest govern ganglia formation in the avian peripheral nervous system? *J. Cell. Biochem.* **27**. 189-203.

Ducibella T, Albertini T, Anderson E and Biggers J. D. (1975). The preimplantation mammalian embryo: characterisations if intercellular junctions and their appearence during development. *Dev. Biol*. **45**. 231-250.

Eccleston P. A, Mirsky R and Jessen K. R. (1991). Spontaneous immortalisation of Schwann cells in culture: short term cultured Schwann cells secrete growth inhibitory activity. *Development*. **112**. 33-42.

Ed. Sherman M. I and Solter D. (1975). *Teratomers and differentiation*. Acad. Press, Inc. New York.

Eghbali B, Kessler J. A and Spray D.C. (1990). Expression of gap junction channels in communication-incompotent cells after stable transfection with cDNA encoding connexin 32. *Proc. Natl. Acad. Sci*. **87**. 1328-1331.

Elfgang C, Eckert R, Lichtenberg H, Butterweck A, Traub O, Klien R. A, Hulser D and Willecke K. (1995). Specific permeability and selective formation of gap junction channels in connexin-transfected HeLa cells. *J. Cell Sci*. **129**. 805-817.

Enomoto T, Martel N, Kanno Y and Yamasaki H. (1984). Inhibition of cell communication between Balb / c3T3 cells by tumour promoters and protection by cAMP. *J. Cell. Physiol.* **121**. 323-333.

Enomoto T and Yamasaki H. (1994). Lack of intercellular communication between chemically transformed and surrounding nontransformed BALB / c3T3 cells. *Cancer Res*. **44**. 5200-5203.

Erickson C. A and Perris R. (1993). The role of cell-cell and cell-matrix interactions in the morphogenesis of the neural crest. *Develop. Biol*. **159**. 60-74.

Esinbuy C. B, Chang C. C, Trosko J. E and Ruch R. J. (1995). *In vitro* growth inhibition of neoplastically transformed cells by non-transformed cells: requiremnet for gap jucntional communication. *Carcinogenesis*. **16**. 915-921.

Etoh T, Thomas L, Levy C. P, Colvin R. B, Mihm M. C and Byers H. R. (1993). Role of integrin $\alpha 2\beta 1$(VLA-1) in the migration of human melanoma cells on laminin and type IV collagen. *J. Investigative Dermatology*. **5**. 640-645.

Faccini A. M, Cairney M, Ashrafi H, Finbow E. M, Campo S and Pitts J. D. (1996). The bovine papillomavirus type 4 E8 protein binds to ductin (16kDa) and causes loss of gap junctional intercellular communication in primary fibroblasts. *Submitted for publication.*

Fawcett D. W and McNutt N. S. (1969). The ultrastructure of the cat myocardium (1). *J. Cell Biol.* **42**. 1-45.

Fernandez J. L, Geiger B, Salomon D, Sabanay I, Zoller M and Ben-Ze'ev A. (1992). Suppression of tumourigenicity in transformed cells after transfection with vinculin cDNA. *J. Cell Biol.* **119**. 427-438.

Fiering S, Roederer M, Herzenberg L.A. (1991). FACS-Gal: Flow cytometric analysis and sorting of cells expressing reporter gene constructs. *Methods: Comp. Meth. Enzymol.* **2**. 261-267.

Filson A. J, Azarnia R, Beyer E. C, Loewenstein W. R and Brugge J. S. (1990). Tyrosine phosphorylation of a gap junction protein correlates with inhibition of cell-cell communication. *Cell growth and differentiation*. **1**. 661-668.

Finbow E. M and Pitts J. D. (1993). Is the gap junction channel - the connexon - made of connexin or ductin. *J. Cell Science.* **106**. 463-472.

Finbow M, Eliopoulos E. E, Jackson P. J, Keen J. N, Meagher L, Thompson P, Jones P and Findlay J. B. (1992). Structure of a 16 kDa integral membrane protein that has identity to the putative proton channel of the vacuolar H- ATPase. *Pro. Eng*. **5**. 7-15

Finbow M. E, John S, Kam E, Apps D and Pitts J. D. (1993). Disposition and orientation of ductin (DCCD-reactive vacuolar H-ATPase Subunit) in mammalian membrane complexes. *Exp. Cell. Res.* **207**. 261-270

Finbow M. E, Buultjens T. E, Lane N. J and Pitts J. D. (1984). Isolation and characterisation of arthropod gap junctions. *EMBO J.* **3**. 2271-2278.

Finbow M. E, Pitts J. D, Goldstein D. J, Schlegel R and Findlay J. B. C. (1991). The E5 oncoportein target: a 16kD channel forming protein with diverse functions. *Mol. Carcinogenesis*. **4**. 441-444.

Finbow M. E and Meagher L. (1992). Connexins and the vacuolar proteolipid-like 16-kDa protein are not directly associated with each other but may be components of similar or the same gap junctional complexes. *Exp. Cell. Res.* **203**. 280-284.

Flagg-Newton J. L, Dahl G and Loewenstein W. R. (1981b). Cell junction and cAMP. I. Up regulation of junction membrane permeability and junctional membrane particles by administration of cyclic nucleotide or phosphodiesterase inhibitor. *J. Mem. Biol.* **63**. 105-121.

Flagg-Newton J. L, and Loewenstein W. R. (1981a). Cell junction and cAMP. II. Modulation of junctional membrane permeability, dependent on serum and cell density. *J. Mem. Biol.* **63**. 123-131.

Fraser S. E, Green C. R, Bode H. R and Gilula N. B. (1987). Selective disruption of gap junctional communication interferes with the patterning process in Hydra. *Science*. **237**. 49-55.

Fraser S. E and Byrant P. J. (1985). Patterns of dye coupling in the imaginal disc of Drosophila melanogaster. *Nature*. **317**. 533-536.

Fujimori T and Takeichi M. (1993). Disruption of epithelial cell-cell adhesion by exogenous expression of a mutated nonfunctional N-cadherin. *Mol. Biol. Cell.* **4**. 37-47.

Gainer H. S and Murray A. W. (1985). Diacylglycerol inhibits gap junctional communication in cultured epidermal cells: evidence for a role of protein kinase C. *Biochem. Biophys. Res. Comm.* **126**. 1109-1113.

Garrod D. R. (1993). Desmosomes and hemidesmosomes. *Current Biology*. **5**. 30-40.

Gavelle F. M and Duband J. L. (1995). Control of N-cadherin intercellular adhesion in migrating neural crest cells in vitro. *J. Cell. Sci.* **108**. 3839-3853.

Geiger B, Salomon D, Takeichi M and Hynes R. O. (1992). A chimeric N-cadherin/β1-integrin receptor which localises to both cell-cell and cell-matrix adhesions. *J. Cell Sci.* **103**. 943-951.

Georges R. N, Mukhopadhyhay T, Zhang Y, Yen N and Roth J. A. (1993). Prevention of orthotopic human lung cancer growth by intracellular instillation of a retroviral antisense K-ras construct. *Cancer Res*. **53**. 1743-1746.

Ghibelli L, Coppola S, Nosseri C, Bergamini A and Beninati S. (1994). A protein produced by a monocytic human cell line can induce apoptosis on tumour cells. *J. Cell Sci*. **344**. 35-40.

Giaume C, Randriamampita C and Trautmann A. (1989). Arachidonic acid closes gap junction channels in rat lacrimal glands. *Eur. J. Physiol*. **413**. 273-279.

Gibson D. F., Hossain M. Z., Goldberg G. S., Acevedo P and Bertram J. S. (1994). The mitogenic effects of transforming growth factor-β1 and β2 in C3H/10T cells occur in the presence of enhanced gap junctional communication. *Cell Growth and Differentiation* 5. 687-696.

Gimilich R. L, Kumar N. M and Gilula N. B. (1990). Differential regulation of the levels of three different gap junction mRNA's in *Xenopus* embryos. *J. Cell. Biol.* **110**. 597-605.

Gimond C and Aumailley M. (1992). Cellular interactions with the extracellular matrix are coupled to diverse transmembrane signalling pathways. *Exp. Cell Res.* **203**. 365-373

Ginsburg M. H, Du X and Plow E. (1992). Inside-out integrin signalling. *Current Biol*. **4**. 766-771.

Girvin M. E and Fillingham R. H. (1993). Hairpin folding of subunit c of the F_1F_0 ATP synthase: ^1H NMR resonance assignments and NOE analysis. *Biochem.* **31.** 12167-12177.

Girvin M. E and Fillingham R. H. (1994). Helicle structure and folding of subunit c of the F_1F_0 ATP synthase: ^1H distance measurements to nitroxide-derivitized aspartyl-61. *Biochem.* **32.** 665-674.

Glass C. K, Devary O. V and Rosenfeld M. G,. (1990). Multiple cell type specific proteins differentially regulate target sequence recognition by the a retenoic acid receptor. *Cell.* **63.** 729-738.

Glukhova M. A and Thiery J. P. (1993). Fibronectin and integrins in development. *Cancer Biology.* **4.** 241-249.

Goldberg. G. S, Martyn K. N and Lau A. L. (1994). A connexin antisense vector reduces the ability of normal cells to inhibit the foci formation of transformed cells. *Mol. Carcinogenesis.* **11.** 106-114.

Goldstein D. J, Kulke R, Dimaio D and Schlegel D. (1992). A glutamine residue in the membrane-associating domain of the bovine papillomavirus type 1 E5 oncoprotein mediates its binding to a transmembrane component of the vacuolar H-ATPase. *J. Virol.* **66.** 405-413.

Goldstein D. J, Andresson T, Sparkowski J. J and Shlcgel D. (1992). The BPV-1 E5 protein, the 16kDa membrane pore-forming protein and the PDGF ral junctional complex. *J. Cell Biology.* **107.** 1575-1587.

Goliger J. A and Paul D. L. (1994). Expression of gap junction proteins Cx26, Cx31, Cx37 and Cx43 in developing and mature rat epidermis. *Dev. Dyn.* **200.** 1-13.

Goliger J. A and Paul D. L. (1995). Wounding alters epidermal connexin expression and gap junction-mediated intercellular communication. *Mol. Biol. Cell.* **6.** 1491-1501.

Goodenugh D. A and Musil L. S. (1994). Gap junctions and tissue business: problems and strategies for developing specific functional reagents. *J. Cell. Sci.* **17.** 133-138.

Gosh S and Baltimore D. (1990). Activation in vitro of NF-$\varkappa$B by phosphorylation of its inhibitor I$\varkappa$B. *Nature.* **344.** 678-682.

Gradl G, Faust D and Weiser R. J. (1995). Density dependent regulation of cell growth by contactinhibin and the contactinhibin receptor. *Current Biology.* **5.** 526-535.

Grana X and Reddy E. P. (1995). Cell cycle control in mammalian cells: role of cyclins, cyclin dependent kinases, growth suppressor genes and cyclin dependent kinase inhibitors. *Oncogene.* **11.** 211-219.

Greenwald I and Rubin G. M. (1995). Making a difference: The role of cell-cell interactions in establishing separate identities for equivalent cells. *Cell.* **68.** 271-281.

Gumbiner B, Stevenson B and Grimaldi A. (1988). The role of the cell adhesion molecule uvomorolin in the formation and maintainence of the epithelial junctional complex. *J. Cell. Biol.* **107.** 1575-1587.

Gumbiner B. M and Yamada K. M. (1992). Cell-to-cell contact and extracellular matrix. *Current Biology*. **4**. 757-759.

Gumbiner B. M. (1987). Structure, biochemistry and assembly of epithelial tight junctions. *Cell Physiol*. **253**. 749-758.

Gumbiner B. M. (1993). Proteins associated with the cytoplasmic surface of adhesion molecules. *Neuron*. **11**. 551-564.

Gumbiner B. M. (1995). Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell*. **84**. 345-357.

Gumbiner B. M. (1996). Signal transduction by β-catenin. *Curr. Opin. Cell Biol*. **7**, in press.

Guo H, Acevedo P, Parsa F. D and Bertrum J. S. (1992). Gap junctional protein connexin 43 is expressed in dermis and epidermis of human skin: differential modulation by retenoids. *Invest. Dermatol*. **99**. 460-467.

Guthrie P. B, Lee R. E, Schmidt M. F and Kater S. B. (1994). Self-recognition: A constraint on the formation of electrical coupling in neurons. *J. Neuroscience*. **14**. 1477-1485.

Hall B. K, Exan V, Brunt S. L. (1983). Retention of epithelial basal lamina allows isolated mandibular mesenchyme to form bone. *J. Craniofac. Genet. & Devl. Biol*. **3**. 253-267.

Hamaguchi M, Matsuyoshi N, Ohnishi Y, Gotoh B, Takeichi M and Nagi Y. (1993). p60v-src causes tyrosine phosphorylation and inactivation of the N-cadherin-catenin cell adhesion system. *EMBO J*. **12**. 307-314.

Hartig E, Nierlich B, Mink S, Nebl G and Cato A. C. B. (1993). Regulation of expression of mouse mammary tumour virus through sequences located in the hormone response element: involvement of cell-cell contact and a negative regulatory factor. *J. Virology*. **67**. 813-821.

Haskill S, Beg A. A, Tompkins S. M, Morris, J. S, Yurochko A. D, Johannes S. A, Mondal K, Ralph P and Baldwin A. S. (1991). Characterisation of an intermediate early gene induced in adherent monocytes that encodes I Kappa B-like activity. *Cell*. **65**. 1281-1289.

Hay E. D. (1981). *Cell biology of extracellular matrix*. New York. Academic press.

Heasman J, Ginsberg D, Geiger B, Goldstone K, Pratt T, Yoshida N. C and Wylie C. (1994). A functional test for maternally inherited cadherin in Xenopus shows its importance in cell adhesion at the blastula stage. *Development*. **120**. 49-57.

Heitzler P and Simpson P. (1991). The choice of cell fate in the epidermis of Drosophila. *Cell*. **64**. 1083.

Helms J. A, Kim C. H, Eichele G and Thaller C. (1996). Retenoic acid signalling is required during early chick limb development. *Development*. **122**. 1385-1394.

Hermiston M. L and Gordon J. I. (1995). In vivo analysis of cadherin function in the mouse intestinal epithelium: Essential roles in adhesion, maintainence of differentiation and regulation of programmed cell death. *J. Cell. Biol.* **129**. 489-506.

Hertig C. M, Butz S, Koch S, Eberhardt M. E, Kemler R and Eppenberger H. M. (1996). N-cadherin in adult rat cardiomyocytes in culture. II. Spatial-temporal appearence of proteins involved in cell-cell contact and communication. *J. Cell Sci.* **109**. 11-20.

Hinck L, Nelson W. J and Papkoff J. (1994). Wnt-1 modulates cell-cell adhesion in mammalian cells by stablising β-catenin binding to the cell adhesion protein cadherin. *J. Cell Biol.* **124**. 729-741.

Hirano S, Kimoto N, Shimoyama Y, Hirohashi S and Takeichi M. (1992). Identification of a neural a-catenin as a key regulator of cadherin function and multicellular organization. *Cell.* **70**. 293-301.

Hirano S, Nose A, Hatta K, Kawakami M and Takeichi M. (1987). Calcium-dependent cell-cell adhesion molecules (cadherins): subclass specificities and possible involvement of actin bundles. J. Cell Bio. *J. Cell Biol.* **105**. 2501-

Hoh J. H, Lal R, John S. A, Revel J. P and Arnsdorf M. F. (1991). Atomic force microscopy and dissection of gap junctions. *Science*. **253**. 1405-1408.

Holden P, Pitts J. D, Stoler A, Canno A and Balmain B. (1996). Changes in gap junctional intercellular communication during progression in mouse skin carcinogenesis. *Submitted for publication - British J. Cancer.*

Holder J. W, Elmore E and Barrett J. C. (1993). Gap junction function and cancer. *Cancer Res.*. **53**. 3475-3485.

Holzenburg A, Jones P. C, Franklin T, Pali T, Heimburg T, Marsh D, Findlay J. B. C and Finbow M. E. (1993). Evidence for a common structure for a class of membrane channel. *Eur. J. Biochem.* **213**. 21-29.

Hong W. K, Lippman S. M, Itri L. M, Karp D. D, Lee J. S, Beyers R. M and Goepfert H. (1990). Prevention of second primary tumours with isotretinoin in squamous-cell carcinoma of the head and neck. *New Engl. J. Med.* **323**. 795-801.

Honn K. V and Tang D. G. (1992). Adhesion molecules and tumour cell interaction with endothelium and subendothelial matrix. *Cancer and Metastasis*. **11**. 353-375.

Hooper M. L and Subak-Sharpe J. H. (1981). Metabolic co-operation between cells. *Int. Rev. Cytol.* **69**. 45-104.

Hortsch M and Goodman C. S. (1991). Cell and substrate adhesion molecules in Drosophila. *Ann. Rev. Cell. Biol.* **7**. 505-557.

Horwitz B. H, Burkhardt A, Schlegel R and Dimaio D. (1988). 44-Amino-acid E5 transforming protein of bovine papillomavirus requires a hydrophobic core and specific carboxyl-terminal amino acids. *Mol. Cell. Biol.* **8**. 4071-4078.

Hsiao W. L. W, Celli S. G and Weinstein I. B. (1984). Oncogene-induced transformation of C3H 10T1/2 cells is enhanced by tumor promoters. *Science.* **226**. 552-555.

Huber B. E, Richards C. A and Krenitsky T. A. (1991). Retroviral-mediated gene therapy for the treatment of hepatocellular carcinoma: An innovative approach for cancer therapy. *Proc. Natl. Acad. Sci.* **88.** 8039-8043.

Huhtala P, Humphries M. J, McCarthy J. B, Tremble P. M, Werb Z and Damsky C. H. (1995). Co-operative signalling by $\alpha 5\beta 1$ integrins regulates metalloproteinase gene expression in fibroblasts adhering to fibronectin. *J. Cell Biol.* **129.** 867-879.

Hulsken J, Birchmeier W and Behrens J. (1994). E-cadherin and APC compete for the interaction with β-catenin and the cytoskeleton. *J. Cell. Biol.*. **127.** 2061-2069.

Husoy T, Mikalsen S. O and Sanner T. (1993). Phosphate inhibitors, gap junctional intercellular communication and [125I]-EGF binding in hamster fibroblasts. *Carcinogenesis*. **14.** 2257-2265.

Hyde S. C, Gill D. R, Higgens C. F and Trezise A. O. (1993). Correction of the ion transport defect in cystic fibrosis transgenic mice by gene therapy. *Nature*. **362.** 250-255.

Hynes R. O. (1992a). Integrins: versatility, modulation and signalling in cell adhesion. *Cell.* **69.** 11-25

Hynes R. O. (1992b). Specificity of cell adhesion in development: the cadherin superfamily. *Current Biology*. **2.** 621-624.

Inagaki M, Moustakas A, Lin H. Y, Lodish H. F and Carr B. I. (1993). Growth inhibition by transforming growth factor-β type 1 is restored in TGF-β-resistant hepatoma cells after expression of TGF-β type 11 cDNA. *Proc. Natl. Acad. Sci.* **90.** 5359-5363.

Ingber D. E, Dike L, Hansen L, Karp S, Liley H, Maniotis A, McNamee H, Mooney D, Plopper G, Sims J and Wang N. (1994). Cellular tensegrity: Exploring how mechanical changes in the cytoskeleton regulate cell growth, migration and tissue pattern during morphogenesis. *Int. Rev. Cytol.* **150.** 173-225.

Ingham P. W. (1994). Pattern formation: hedgehog points the way. *Curr. Biol.* **4.** 374-380.

Itoh M, Nagafuchi A, Yonemura S, Yasuda T. K, Tsukita S and Tsukita S. (1993). The 220kD protein colocalizing with cadherins in non-epithelial cells is identical to ZO-1, a tight junction-associated protein in epithelial cells: cDNA cloning and immunoelectron microscopy. *J. Cell. Biol.* **121.** 491-502.

Jaonnou M.K, Woolf A.S and Henderson R. M. (1995). The hepatocyte growth factor / scatter factor (HGF/SF) receptor, met, transduces a morphogenetic signal in renal glomerular fibromuscular mesangial cells. *J. Cell Sci.* **108.** 3703-3714.

Jaslove S. W and Brink P. R. (1986). The mechanism of rectification at the electronic motor giant synapse of the crayfish. *Nature*. **323.** 63-65.

Javed Q, Fleming T. P, Hay M and Citi S. (1993). Tight junction protein cingulin is expressed by maternal and embryonic genomes during early mouse development. *Development*. **117.** 1145-1151.

Jessel T. M, and Melton D. A. (1992). Diffusible factors in vertebrate embryonic induction. *Cell.* **68.** 257-270.

Jiang T. X and Chuong C. M. (1992). Mechanism of feather morphogenesis: I. Analysis with antibodies to adhesion molecules tenascin, N-CAM and integrin. *Dev. Biol.* **150.** 82-98.

Johnston R. L and Tabin C. (1995). The long and short of hedgehog signalling. *Cell.* **81.** 313-316.

Jongen W. F, Fitzgerald J. D, Asamoto M, Piccoli C, Slaga T. J, Gros D, Takeichi M and Yamasaki H. (1991). Regulation of connexin 43- mediated gap junctional intercellular communication by Ca^{2+} in mouse epidermal cells is controlled by E-Cadherin. *J. Cell. Biol.* **114.** 545-555.

Jou Y. S, Matesic D, Dupont E, Lu , S. C, Rupp H. L, Trosko J. E and Chang C. C. (1993). Restoration of gap junctional intercellular communication in a communication defeicient rat liver cell mutant by transfection with connexins43 cDNA. *Mol. Carcinogensis.* **8.** 234-244.

Juliano R. L and Haskil S. (1993). Signal transduction from the extracellular matrix. *J. Cell. Biol.* **120.** 577-585.

Juliano R. L and Varner J. A. (1993). Adhesion molecules in cancer: the role of integrins. *Current Biology.* **5.** 812-818.

Kalimi G. H, Hampton L.L, Trosko J. E, Thorgeirsson S. S and Huggett A. C. (1992). Homologous and heterologous gap junctional intercellular communication in v-raf, v-myc and v-raf/v-myc transduced rat liver epithelial cell lines. *Molecular Carcinogenesis.* **5.** 301-310.

Kalimi G. H and Lo C. W. (1988). Communication compartments in the gastrulating mouse embryo. *J. Cell Biol.* **107.** 748-753.

Kam E and Hodgins M. B. (1992). Communication compartments in hair follicles and their implication in differentiative control. *Development.* **114.** 389-393.

Kam E and Pitts J. D. (1988). Effects of the tumour promoter 12-0-TPA-13-acetate on junctional communication in intact mouse skin: persistence of homologous communication and increase of epidermal-dermal coupling. *Carcinogenesis.* **9.** 1389-1394.

Kam E. Melville L and Pitts J. D. (1986). Patterns of junctional communication in skin. *J. Invest. Derm.* **87.** 748-753.

Kamibayashi Y, Oyamada Y, Mori M and Oyamada. (1995). Aberrant expression of gap junction proteins (connexins) is associated with tumour progression during multistage carcinogenesis in vivo. *Carcinogenesis.* **16.** 1287-1297.

Kanemitsu M. Y and Lau A. F. (1993). Epidermal growth factor stimulates the disruption of gap junctional communication and connexin 43 phosphorylation independent of 12-0-TPA-sensitive protein kinase C: the possible involvement of mitogen activated protein kinase. *Mol. Biol. Cell.* **4.** 837-848.

Kanner S. B, Grosmaire L. S, Ledbetter J. A and Damle N. K. (1993). β2-integrin LFA-1 signalling through phospholipase C-γ1 activation. *Proc. Natl. Acad. Sci.* **90**. 7099-7103.

Katoh F, Klein J, Bignami M and Yamasaki H. (1993) Association of viral oncogene-induced changes in gap junctional intercellular communication and morphological transformation in BALB/c3T3 cells. *Carcinogenesis*. **14**. 3. 435-440.

Keane R. W, Mehta P. P, Rose B, Honig L. S, Loewenstein W. R and Rutishauser U. (1988). Neural differentiation, N-CAM mediated adhesion and gap junctional communication in neuroectoderm. A study *in vitro*. *J. Cell Biol.* **106**. 1307-1319.

Kerr W. G and Herzenberg L. A. (1991). Gene-search viruses and FACS-Gal permit the detection, isolation and characterisation of mammalian cells with *in situ* fusions between cellular genes and *E. coli lacZ. Methods: Comp. Meth. Enzymol.* **2**. 261-271.

Kinch M. S, Clark G. J, Der C. J and Burridge K. (1995). Tyrosine phosphorylation regulates the adhesions of Ras transformed breast epithelia. *J. Cell. Biol.* **130**. 461-471.

Klagsburn M. (1990). The affinity of fibroblast growth factors for heparin: FGF-heparan sulphate interactions in cells and ECM. *Curr. Opin. Cell Biol.* **2**. 857-863.

Klann R. C, Fitzgerald D. J, Piccoli C, Slaga T. J and Yamasaki H. (1989). Gap-junctional intercellular communication in epidermal cell lines derived from selected stages of SENCAR mouse skin carcinogenesis. *Cancer Res.* **49**. 699-705.

Klasgburn M and Baird A. (1991). A dual receptor system is required for fibroblast growth factor activity. *Cell*. **67**. 229-231.

Klaunig J. E and Ruch R, J, (1990). Biology of disease: role of inhibition in intercellular communication in carcinogenesis. *Lab. Ivest*. **62**. 135-146.

Klein G, Bregula U, Weiner T and Harris H. (1971). The analysis of malignancy by cell fusion. I. Hybrids between tumour cells and L cell derivatives. *J. Cell Sci.* **8**. 659-672.

Kleinsmith E. J and Pierce G. B. (1964). Multipotentiality of single embryomal carcinoma cells. *Cancer*. **24**. 1544-1552.

Konig N and Zampighi G. A. (1995). Purification of bovine lens cell-cell channels composed of connexin44 and connexin50. *J. Cell Sci.* **108**. 3091-3098.

Konshi T. (1990). Dye coupling between mouse schwann cells. *Brain Res.* **508**. 85-92.

Kornberg L, Earp H. S, Turner C, Prokop C and Juliano R. L. (1991). Signal transduction by integrins: increased protein tyrosine phosphorylation caused by clustering of b1 integrins. *Proc. Natl. Acad. Sci.*. **88**. 8392-8396.

Koskinen P. J, Sistone L, Bravo R and Alitalo K. (1991). Immediate early gene responses of NIH3T3 fibroblasts to TGF-β1. *Growth Factors*. **5**. 283-293.

Kramer H, Cagan R. L and Zipursky S. L. (1991). Interaction of bride of sevenless membrane-bound ligand and the sevenless tyrosine kinase receptor. *Nature*. **352**. 207-212.

Krutovskikh V. A, Oyamada M and Yamasaki H. (1991). Sequential changes of gap junctional intercellular communication during multi-stage rat liver carcinogenesis: direct measurement of communication in vivo. *Carcinogenesis*. **12**. 1701-1706.

Kumar N. M, Friend D.S and Gilula N. B. (1995). Synthesis and assembly of human β1 gap junctions in BHK cells by DNA transfection with the human β1 cDNA. *J. Cell. Sci.* **108**. 3725-3734.

Kumar N. M and Gilula N. B. (1992). Molecular biology and genetics of gap junction channels. *Seminars in Cell Biol.* **3**. 3-16.

Kumar N. M. (1991). Gap junctions: A multigene family. *Advs. Struc. Biol.* **1**. 209-247.

Kundson A. G. (1973). *Annu. Cancer Res.* **17**. 317-321.

La Rocca S, Grossi M, Falcone G and Tato F. (1989). Interaction with normal cells suppresses the transformed phenotype of v-myc-transformed Quail muscle cells. *Cell.* **58**. 123-131.

LaFlamme S. E, Akiyama S. K and Yamada K. M. (1992). Regulation of fibronectin receptor distribution. *J. Cell Biol.* **117**. 437-447.

Laird D. W, Puranam K. L and Revel J. P. (1991). Turnover and phosphorylation dynamics of connexin43 gap junction protein in cultured cardiac myocytes. *Biochem. J.* **273**. 67-72.

Lampe P. D. (1994). Analysing phorbol ester effects on gap junctional communication: A dramatic inhibition of assembly. *J. Cell. Biol.*. **127**. 1895-1905

Lau A. F, Piggot V and Crow D.S. (1991). Evidence that heart connexin 43 is a phosphoprotein. *J. Mol. Cell. Cardiol.* **23**. 659-663.

Layton M. G and Franks L. M. (1984). Heterogeneity in a spontaneous mouse lung carcinoma: selection and characterisation of stable metastatic variants. *Br. J. Cancer.* **49**. 415-421.

Layton M. G and Franks L. M. (1986). Selective suppression of metastasis but not tumorigenicity of a mouse lung carcinoma by cell hybridization. *Int. J. Cancer* **57**. 123-130.

Lecuit T, Brook W. J, Ng M, Calleja M, Sun H and Cohen S. M. (1996). Two distinct mechanisms for long-range patterning by decapentaplegic in the drospophila wing. *Nature*. **381**. 387-393.

Lee S, Gilula N. B and Warner A. E. (1987). Gap junctional communication and compaction during preimplantation stages of mouse development. *Cell.* **51**. 851-860.

Lee S. W, Tomasetto C, Paul D, Keyomarsi K and Sager R. (1992). Transcriptional downregulation of gap junction protein blocks junctional communication in human mammary tumour cell lines. *J. Cell. Biol.* **118**. 1213-1221

Lee S. W, Tomasetto C, and Sager R. (1991). Positive selection of candidate tumour suppressor genes by subtractive hybridisation. *Proc. Natl. Acad. Sci.* **88**. 2825-2829.

Leibold E and Shwartz L. R. (1993). Intercellular communication in primary cultures of putative preneoplastic and normal hepatocytes. *Carcinogenesis*. **14**. 2127-2129.

Leitch B and Finbow M. E. (1990). The gap junction-like form of a vacuolar proton channel appears not to be an artefact of isolation:an immunocytochemical localisation study. *Exp. Cell Res.* **190**. 218-226.

Levine E, Lee C. H, Kintner C and Gumbiner B. (1994). Selective disruption of E-cadherin function in early Xenopus embryos by a dominant negative mutant. *Development*. **120**. 901-909.

Lin J-W and Faber D. S. (1988). Synaptic trasmissions mediated by single club endings on the goldfish Mauthner cells. *J. Neuroscience*. **8**. 1302-1312.

Linnainmaa K, Pelin K, Vanhala E, Tuomi T, Piccoli, Fitzgerald D. J and Yamasaki H. (1993). Gap junctional intercellular communication of primary and asbestos-associated malignant human mesothelial cells. *Carcinogenesis*. **14**. 1597-1602.

Linzer D. I. H and Wilder E. L. (1987). Control of proliferin gene expression in serum-stimulated mouse cells. *Mol. Cell. Biol.* **7**. 2080-2086.

Liu L, Blat C and Harel L.(1992). Synthesis and secretion by mouse 3T3 cells of an inhibitor of cell growth (mIGFBP-3): correlation with cell proliferation. *Biol. Cell.* **76**. 125-130.

Lo C. W and Gilula N. B. (1979a). Gap junctional communication in the preimplantation mouse embryo. *Cell*. **18**. 399-409.

Lo C. W. and Gilula N. B. (1979b). Gap junctional communication in the postimplantation mouse embryo. *Cell*. **18**. 411-422.

Lo S. H and Chen L. B. (1994). Focal adhesion as a signal transduction organelle. *Cancer Metastas. Rev*. **13**. 9-24.

Lo W. K and Reese T. S. (1993). Multiple structural types of gap junctions in mouse lens. *J. Cell. Sci*. **106**. 227-235.

Loef E. B, Proper J. A, Goustin A.S, Shipley G. D and Moses H. L. (1989). Induction of c-sis mRNA and activity similar to PDGF by transforming growth factor-β: a proposed model for indirect mitogenesis involving autocrine activity. *Proc. Natl. Acad. Sci.* **83**. 2453-2457.

Loewenstien W. R. (1979). Junctional intercellular communication and the control of growth. *Biochemica Biophysica Acta.* **560**. 1-65.

Luna E. J and Hitt A. L. (1992). Cytoskeleton-plasma membrane interactions. *Science.* **258**. 955-962.

MacDonald C. (1985). Gap junctions and cell-cell communication. *Essays Biochem*. **21**. 86-118.

Maden M. (1982). Vitamin A and pattern formation in the regenerating limb. *Nature*. **295**. 672-675.

Maden M. (1983). The effect of vitamin A on limb regeneration in Rana temporaria. *Devel. Biol.* **98**. 409-416.

Madhukar B. V, Oh S. Y, Chang C. C, Wade M and Trosko J. E. (1989). Altered regulation of intercellular communication by epidermal growth factor, transforming growth factor-β and peptide hormones in normal keratinocytes. *Carcinogenesis*. **10**. 13-20.

Magee A. I and Buxton A. S. (1991). Transmembrane assembilies regulated by the greater cadherin family. *Curr. Opin. Cell Biol.* **3**. 854-861.

Magnuson T, Demsey A and Stackpole C. W. (1977). Characterisation of intercellular junctions in the preimplantation mouse embryo by freeze fracture and thin section electron microscopy. *Dev. Biol.* **61**. 252-261.

Mahmood R, Bresnick J, Hornbuch A, Mahony C, Morton N, Colquhon K, Martin P, Lumsden A, Dickson C and Mason I. (1995). A role for FGF-8 in the intiation and maintainence of vertabrate limb bud outgrowth. *Current Biol.* **5**. 797-806.

Maines J. Z, Sunnarborg A and Boyd F. T. (1995) Positive selection of growth inhibitory genes. *Cell Growth & differentiation*. **6**. 665-671.

Makowski L, Caspar D. L. D, Phillips W. C, Baker T. S and Goodenough D. A. (1984). Gap junction structures. VI. Variation and conservation in connexon conformation and packing. *Biophys. J.* **45**. 208-218.

Makowski L, Caspar D. L. D, Phillips W. C and Goodenough D. A. (1977). Gap junction structures: II analysis of X-ray diffraction data. *J. Cell Biol.* **74**. 629-645.

Makowski L. (1988). X-ray diffraction studies of gap junction structure. *Advanc. Cell Biol.* **2**. 119-158.

Maldonado P. B, Rose B and Loewenstein W. R. (1988). Growth factors modulate junctional cell-cell communciation. *J. Mem. Biol.* **106**. 203-210.

Manabe T, Yoshimori T, Henomatsu N and Tashiro Y. (1993). Inhibitors of vacuolar-type H- ATPase suppresses proliferation of cultured cells. *J. Cell. Physiol.*. **157**. 445-452.

Mandel M, Moriyama Y, Hulmes J. D, Pan Y. C, Nelson H and Nelson N. (1988). cDNA sequence encoding the 16kDa proteolipid of chromaffin granules implies gene duplication in the evolution of H+ATPases. *Proc. Natl. Acad. Sci.* **85**. 5521-5524.

Marrs J. A, Andersoson-Fisone C, Jeong M. C, Gould L. C, Zurzola C, Nabi I. R, Boulan E. R and Nelson J. W. (1995). Plasticity in epithelial cell phenotype: Modulation by expression of different cadherin cell adhesion molecules. *J. Cell. Biol.* **129**. 507-519.

Martin W, Zemple G, Hulser D and Willecke K. (1991). Growth inhibition of oncogene-transformed rat fibroblasts by cocultured normal cells: relevance of metabolic cooperation mediated by gap junctions. *Cancer Res.* **51**. 5348-5354

Martinez S, Geijo E, Vives M. V. S, Puelles L and Gallego R. (1992). Reduced junctional permeability at interhombomeric boundaries. *Development*. **116**. 1069-1076.

Massague J and Pandiella A. (1993). Membrane-anchored growth factors. *Annu. Rev. Biochem.* **62**. 515-541.

Massague J. (1990). Transforming growth factor-α: a model for membrane-anchored growth factors. *J. Biol. Chem.* **265**. 21393-21396.

Matesic D. F, Rupp H. L, Bonney W. J Ruch R. J and Trosko J. E. (1994). Changes in gap junction permeability, phosphorylation and non-phorbol-ester tumor promters in rat liver epithelial cells. *Molecular Carconigensis*. **10**. 226-236.

Matsuura K, Kawanishi J, Fujii S, Imamura M, Hirano S, Takeichi M and Niitsu Y. (1992). Altered expression of E-cadherin in gastric cancer tissues and carcinomtous fluid. *Br. J. Cancer.*. **66**. 1122-1130

Matsuyoshi N, Hamaguchi M, Taniguchi S, Nagafuchi A, Tsukita S and Takeichi M. (1992). Cadherin-mediated cell-cell adhesion is peturbed by v-src tyrosine phosphorylation in metastatic fibroblasts. *J. Cell. Biol.*. **118**. 7-03-714.

McNeill H, Ozawa M, Kemler R and Nelson W. J. (1990). Novel function of the cell adhesion molecule uvomorulin as an inducer of cell surface polarity. *Cell.* **62**. 309-316.

McNeill H, Ryan T. A, Smith S. J and Nelson W. J. (1993). Spatial and temporal dissection of immediate and early events following cadherin-mediated epithelial cell adhesion. *J. Cell. Biol.* **120**. 1217-1226.

Mege R. M, Goudou D, Diaz C, Niclet M, Garcia L, Geraud G and Rieger F. (1992). N-cadherin and N-CAM in myoblast fusion: compared localisation and effect of blockade by peptides and antibodies. *J. Cell Science.* **103**. 897-906.

Mege R. M, Matsuzaki F, Gallin W. J, Goldberg J. I, Cunningham B. A and Edelman G. M. (1988). Contruction of epithelioid sheets by transfection of mouse sarcoma cells with cDNAs for chicken cell adhesion molecules. *Proc. Natl. Acad. Sci.* **85**. 7274-7278.

Mehta P, Bertram J. S and Loewenstein W. R. (1986). Growth inhibition of transformed cells correlates with their junctional communication with normal cells. *Cell.* **44**. 187-186.

Mehta P. P, Yamamoto M and Rose B. (1992). Transcription of the gene for the gap junctional protein connexin 43 and expression of functional cell-cell channels are regulated by cAMP. *Mol. Biol. Cell.* **3**. 839-850.

Mehta P. P, Wagenblatt A. H, Rose B, Shalloway and Loewenstein W. R. (1991a). Incorporation of the gene for a cell-cell channel protein into transformed cells leads to normalization of growth. *J. Mem. Biol.*. **124**. 207-225.

Mehta P. P and Loewenstein W. R. (1991b). Differential regulation of communication by retenoic acid in homologous and heterologous junctions between normal and transformed cells. *J. Cell. Biol.* **113**. 371-379.

Mehta P.P, Bertram J. S and Loewenstein W. R. (1989). The actions of retenoids on cellular growth correlate with their actions on gap junctional communication. *J. Cell Biol.* **108**. 1053-1065.

Mensil M and Yamasaki H. (1993). Cell-cell communication and growth control of normal and cancer cells: Evidence and hypothesis. *Mol. Carcin.* 7. 14-17

Mesnil M, Fraslin J. M, Piccoli C, Yamasaki H and Guillouzo G. (1987). Cell contact but not junctional communication with nilary epithelial cells is required for hepatocytes to maintain differentiated functions. *Exp. Cell. Res.* **173.** 524-533.

Meyer R. A, Laird D. W, Revel J. P and Johnson R. G. (1992). Inhibition of gap junction and adherens junction assembly by connexin and A-CAM antibodies. *J. Cell. Biol.* **119.** 179-189.

Mikalsen S. O. (1993). Heterologous gap junctional intercellular communication in normal and morphologically transformed colonies of Syrian hamster embryo cells. *Carcinogenesis*. **14.** 2085-2090.

Miller T, Dahl G and Warner R. (1988). Structure of gap junction gene: rat connexin32. *Biosci. Rep*. 8. 455-464.

Minkoff R, Parker S.B and Hertzberg E.L. (1991). Analysis of distribution patterns of gap junctions during development of embryonic chick facial and brain. *Development*. **111.** 509-522.

Mintz B and Illmensee K. (1975). Normal genetically mosaic mice produced from malignant teratocarcinomacells. *Proc. Natl. Acad. Sci.* **72.** 3585-3589.

Miyatani S, Shimamura K, Hatta M, Nagafuchi A, Nose A, Matsunaga M, Hatta K and Takeichi M. (1989). Neural cadherin: role in selective cell-cell adhesion. *Science.* **245.** 631-635.

Monsky W. L and Chen W. T. (1993). Proteases of cell adhesion proteins in cancer. *Cancer Biology*. **4.** 251-258

Mosher D. F, Sottile J, Wu C and McDonald J. A. (1992). Assembly of extracellular matrix. *Curr. Opin. Cell Biol.* **4.** 810-818.

Mosher D. F, Sottile J, Wu C and Mcdonald J. A. (1992). Assembly of extracellular matrix. *Curr. Opin. Cell Biol.* **4.** 810-818.

Musil L. S, Cunningham B. A, Edleman G. M and Goodenough D.A. (1990). Differential phosphorylation of the gap junction protein connexin 43 in junctional communication-compotent and - deficient cell lines. *J. Cell. Biol.* **111.** 2077-2088.

Musil L. S and Goodenough D. A. (1991). Biochemical analysis of connexin43 intracellular transport, phosphorylation and assembly into gap junction plaques. *J. Cell. Biol.* **115.** 1357-1374.

Nagafuchi A, Shirayoshi Y, Okazaki K, Yasuda K and Takeichi M. (1987). Transformation of cell adhesion properties by exogenously introduced E-cadherin cDNA. *Nature*. **329.** 341-342.

Nakagawa S and Takeichi M. (1995). Neural crest cell-cell adhesion controlled by sequential and subpopulation-specific expression of novel cadherins. *Development*. **121.** 1321-1332.

Naus C. C, Bechberger J. F, Caveney, S and Wilson J. X. (1991). Expression of gap junction genes in astrocytes and C6 glioma cells. 122. 31-36.

Naus C. C. G, Hearn S, Zhu D, Nicholson B. J and Shivers R. R. (1993). Ultrastructure analysis of gap junctions in C6 glioma cells transfected with connexin 43 cDNA. *Exp. Cell. Res.* **206.** 72-84.

Navarro P, Gomez M, Pizarro A, Gamallo C, Quintanilla M and Cano A. (1991). A role for the E-Cadherin cell-cell adhesion molecule during tumor progression of mouse epidermal carcinogenesis. *J. Cell. Biol.*. **115.** 517-533.

Navarro P, Lozano E and Cano A. (1993). Expression of E- or P-cadherin is not sufficient to modify the morphology and the tumorigenic behavior of murine spindle carcinoma cells: possible involvement of plakoglobin. *J. Cell. Science.* **105.** 923-934.

Nellen D, Burke R, Struhl G and Basler K. (1996). Direct and long-range action of a DPP morphogen gradient. *Cell.* **85.** 357-368.

Neyton J and Trautmann A. (1985). Single channel currents of an intercellular junction. *Nature.* **317.** 331-335.

Nicolson B, Dermietzel R, Teplow D, Traub O, Willecke K and Revel J. P. (1987). Hepatic gap junctions are comprised of two homologous proteins of MW 28000 and 21000. *Nature.* **329.** 732-734.

Nicolson G. L. (1987). Tumour cell instability, diversification and progression to the metastatic phenotype: from oncogene to oncofetal expression. *Cancer Res.* **47.** 1473-1487.

Nigglie E, Rudisuli A, Maurer P and Weingart R. (1989). Effects of general anesthetics on current flow across junctional and non-junctional membranes and contractility in guinea pig myocytes. *Am. J. Physiol.* **256.** 273-281.

Nishi M, Kumar N. M and Gilula N. B. (1991). Developmental regulation of gap junction gene expression during mouse embryonic development. *Developmental Biol.* **146.** 117-130.

Nori M, L'Allemain G and Weber M. J. (1992). Regulation of tetradecanoyl phorbol acetate-induced responses in NIH-3T3 cells by GAP, the GTPase-activating protein associated with $p21^{c\text{-}ras}$. *Mol. Cell Biol.* **12.** 936-945.

Nose A, Nagafuchi A and Takeichi M. (1988). Expressed recombinant cadherins mediate cell sorting in model systems. *Cell.* **54.** 993-1001.

Novelli M. R, Williamson J. A, Tomlinson I. P. M, Elia G, Hodgson S. V, Talbot I. C, Bodmer W. F and Wright N. A. (1996). Polyclonal origin of colonic adenomas in an X0/Xy patient with FAP. *Science.* **272.** 1187-1190.

O'Neill C, Jordan P and Ireland G. (1986). Evidence for two distinct mechanisms of anchorage stimulation in freshly explanted and 3T3 mouse fibroblasts. Cell 44. 489-496.

Obata S, Sago H and Suzuki S. T. (1995). Protocadherin Pcdh2 shows properties similar to, but distinct from, those of classical cadherins. *J. Cell Sci.* **108.** 3765-3773

Oda H, Uemura T, Shiomi K, Nagafuchi A, Tsukita S and Takeichi M. (1993). Identification of a drosophila homologue of α-catenin and its association with the armadillo protein. *J. Cell. Biol.*. **121**. 1133-1140.

Olsen B. R. (1995). New insights into the functions of collagens from genetic analysis. *Current Opinion in Cell Biol.* **7**. 55-59.

Olsen D. J and Moon R. T. (1992). Distinct effects of ectopic expression of Wnt-1, activin B and bFGF on gap junctional permeability in 32-cell Xenopus embyos. *Developmental Biology*. **151**. 204-212.

Olsen D. J, Christian J. L and Moon R. T. (1991). Effect of wnt-1 and related proteins on gap junctional communication in Xenopus embryos. *Science*. **252**. 1137-1176.

Oyamada M, Krutovskikh V. A, Mensil M, Partensky C, Berger F and Yamasaki H. (1990). Aberrant expression of gap junction gene in primary human hepatocellular carcinomas: increased expression of cardiac-type gap junction gene connexin 43. *Molecular Carcinogenesis*. **3**. 273-278.

Ozawa M and Kemler R. (1992). Molecular organization of the uvomorolin-catenin complex. *J. Cell. Biol.* **116**. 989-996.

Patel P and Lupski J. R. (1994). Charcot-Marie-Tooth disease: a new paradigm for the mechanism of inherited disease. *Trends in Genetics.* **10**. 128-132.

Paul D. L, Bruzzone R, Glimch R. L and Goodenough D. A. (1995). Expression of a dominant negative inhibitior of intercellular communication in the early Xenopus embryo causes delamination and extrusion of cells. *Development*. **121**. 371-381.

Paul D. L. (1985). Antibody against liver gap junction 27-kD protein in tissue specific and cross-reacts with a 54-kD protein. In: *Gap junctions.* Ed Bennett M. V. L and Spray D. C. Cold Spring Harbour Press. 107-122.

Peifer D. L. (1995). Cell adhesion and signal transduction: the Armadillo connection. *Trends Cell. Biol.* **5**. 224-229.

Pierce G. B, Dixon F. J and Verney E. L. (1960). Teratocarinogenic and tissue forming potentials of the cell types comprising neoplastic embryoid bodies. *Lab Invest.* **9** 583-602.

Pierce G. B and Dixon F. J. (1959). Testicular teratomas : demonstration of teratogenesis by metamorphosis of multipotentail cells. Cancer. **12**. 573-583.

Pierce G. B. (1967). Teratocarcinoma: a model for a developmental concept of cancer. *Curr. Top. Devlop. Biol.* **2**. 223-240.

Pim D, Collins M and Banks L. (1992). Human papillomavirus type 16 E5 gene stimulates the transforming activity of the epidermal growth factor receptor. *Oncogene*. **7**. 27-32.

Pitts J. D, Kam E, Melville L and Watt F. M. (1986). Patterns of junctional communication in animal tissues. Junctional Complexes of epithelial cells. *Ciba Foundation Symposium.* **125**. 140-153.

Pitts J. D, Kam E and Morgan D. (1988). The role of gap junctional communication in growth control and tumorigenesis. In *Gap junctions.* ed. Hertzberg E. L and Johnson R. G. pp 397-409. Alan Liss Inc. New York.

Pitts J. D and Burk R. R. (1976). Specificity of junctional communication between animal cells. *Nature.* **264.** 760-762.

Pitts J. D and Kam E. (1985). Communication compartments in mixed cell cultures. *Exp. Cell. Res.* **156.** 439-449.

Pitts J. D and Simms J. W. (1977). Permeability of junctions between animal cells. Intercellular transfer of nucleotides but not of macromolecules. *Exp. Cell Res.* **156.** 439-449.

Pitts J. D. (1971). Molecular exchange and growth control in tissue culture. In *Growth Control in Cell Cultures*, pp89-105. Ciba Foundation Symposium.

Pitts J. D. (1980). The role of junctional communication in animal tissues. *In Vitro.* **16.** 1049-1056.

Prowse D. (1993) Gap junction specificity. PhD Thesis.

Ram Z, Culver K. W, Wallbridge S, Blaese R. M and Oldfield E. H. (1993). In situ retroviral-mediated gene transfer for the treatment of brain tumours in rats. *Cancer Res.* **53.** 83-88.

Rapraeger A. C, Krufka A and Olwin B. B. (1991). Requirement of heperan sulphate for βFGF-mediated fibroblast growth and myoblast differentiation. Science. 252. 1705-1708.

Reaume A. G, Sousa P. A, Kulkarni B, Langille B. L, Zhu D, Davies T. C, Juneja S. C, Kidder G. M and Rossant J. (1995). Cardiac malformation in neonatal mice lacking connexin43. *Science.* **261.** 1831-1834.

Reszka A. A, Hayashi Y and Horwitz A. F. (1992). Identification of amino acid sequences in the integrin β1 ctyoplasmic domain implicated in cytoskeletal association. *J. Cell Biol.* **117.** 1321-1330.

Reynhout J. K, Lampe P. D and Johnson R. G. (1992). An activator of protein kinase C inhibits gap junction communication between cultured bovine lens cells. *Exp. Cell. Res.* **198.** 337-342.

Rimm D. L, Koslov E. R, Kebriaei P, Cianci C. D and Morrow J. S. (1995). α1 (E)-catenin is an actin binding and bundling protein mediating the attachment of F-actin to the membrane adhseion complex. *Proc. Natl. Acad. Sci.* **92.** 8813-8817.

Risek B, Klier F. G and Gilula N. B. (1992). Multiple gap junction genes are utilised during rat skin and hair development. *Development.* **116.** 639-651.

Risek B and Gilula N. B. (1991). Spatiotemporal expression of three gap junction gene products involved in fetomaternal communication during rat pregnancy. *Development.* **113.** 165-181.

Robinson S. R, Hampson E. C. G. M, Munro M. N and Vaney D. I. (1993). Unidirectional coupling of gap junctions between neuroglia. *Science.* **262.** 1072-1074.

Rodriguez E. B and Nelson W. J. (1989). *Science.* **245.** 718-721.

Rogers M, Berestecky J. M, Hossain M. Z, Guo H, Kadle R, Nicholson B. J and Bertrum J. S. (1990). Retenoid-enhanced gap junctional communication is achieved by increased levels of connexin 43 mRNA and protein. *Molecular Carcinogenesis.* **3.** 335-343.

Rook M. B, de Jong B, Jongsma H. J and Masson-Pevet M. A. (1990). Gap junction formation and functional interaction between rat cardiocytes in culture: a correlative physiological ultrastructural study. *J. Mem. Biol.* **118.** 179-192.

Rose B, Simpson I and Loewenstein W. R.(1977). Calcium ion produces graded changes in permeability of membrane channels in cell junction. *Nature.* **267.** 625-627.

Rose B, Mehta P. P and Loewenstein W. R. (1993). Gap junction protein gene suppresses tumorigenicity. *Carcinogenesis.* **14.** 1073-1075.

Rose B and Rick R. (1978). Intracellular pH, intracellular free Ca and junctional cell-cell coupling. *J. Mem. Biol.* **44.** 377-415.

Rosenbaum J, Mavier P, Preaux A. M, Lescs M. C and Dhumeaux D. (1989). Mouse hepatic endothelial cells in culture secrete a growth inhibitor for hepatic lipocytes and balb/c3T3 fibroblasts. *J. Hepatology.* **9.** 295-300.

Rosenburg M. W and Massague J. (1993). Juxtacrine signalling molecules. *Curr. Opin. Cell Biol.* **5.** 832-838.

Roseng L. E, Rivedal E and Sanner T. (1993). 12-*O*-Tetradecanoylphorbol-13-acetate-induced inhibition of gap junctional communication is differentially regulated in a transformation-sensitive Syrian hamster embryo cell line compared to early passage SHE cells. *Carcinogenesis.* **14.** 1851-1855.

Rubin H. (1993). Cellular epigenetics: effects of passage history on competence of cells for "spontaneous" transformation. *Proc. Natl. Acad. Sci.* **90.** 10715-10719.

Rubin H. (1994). Cellular epigenetics: controlof the size, shape and spatial relationship between the transformed and nontransformed cells. *Proc. Natl. Acad. Sci.* **91.** 1039-1043.

Rubin L. L. (1992). Endothelial cells: adhesion and tight junctions. *Current Opinion in Cell Biol.* **4.** 830-833.

Ruch R. J, Madhukar B. V, Trosko J. E and Klaunig J. E. (1993). Reversal of ras-induced inhibition of gap junctional intercellular communication, transformation and tumorigenesis by lovastatin. *Molecular Carcinogenesis.* **7.** 50-59.

Sabban M. E and Pauli B. U. (1991). Cytoplasmic dye transfer between metastatic tumour cells and vascular endotheilium. *J. Cell. Biol.* **115.** 1375-1382.

Saez J. C, Nairn A. C, Czernik A. J, Spray D. C, Hertzberg E. L, Greengard P and Bennett M. V. (1990). Phosphorylation of connexin 32, a hepatocyte gap-junction

protein, by cAMP-dependent protein kinase, protein kinase C and Ca++ / calmodulin-dependent protein kinase II. *Eur. J. Biochem.* **192.** 263-273.

Saez J. C, Connor J. A, Spray D.C and Bennett M. V. (1989). Hepatocyte gap junctions are permeable to the second messenger, inositol 1,4,5-triphosphate and to calcium. *Proc. Nat. Acad. Sci.* **86.** 2708-2712.

Saez J. C, Spray D. C, Nairn A. C, Hertzberg E, Greengard P and Bennett M. V. L. (1986). cAMP increases junctional conductance and stimulates phosphorylation of the 27-kDa principle gap junction polypeptide. *Proc. Natl. Acad. Sci.* **83.** 2473-2477.

Sager R. (1985). Genetic suppression of tumour formation. *Adv. Cancer Res.* **44.** 43-67.

Santoni M. J. Barthesis D, Vopper G, Boned A, Goridis C and Willie W. (1989). Differential exon usage involving an unsual splicing mechanism generates at least eight types of NCAM cDNA in mouse brain. *EMBO J.* **8.** 385-392.

Sawyer R. H and Falon J. F. (1983). *Epithelial-mesenchymal interactions in development.* New York. Praeger Publishers.

Schiller P. C, Mehta P, Roos P and Howard G. A. (1992). Hormonal regulation of intercellular communication: parathyroid hormone increases Cx43 gene expression and gap junctional communication in osteoblastic cells. *Mol. Endocrinol.* **6.** 1433-1440.

Schmid T. M and Linsenmayer T. F. (1985). Developmental acqusition of type X collagen in the embryonic chick tibiotarsus. *Devel. Biol.* **107.** 373-381.

Sefton M, Johnson M. H and Clayton L. (1992). Synthesis and phosphorylation of uvomorulin during mouse early development. *Development.* **115.** 313-318.

Segal S.S. and Beny J. L. (1992). Intracellular recording and dye transfer in arterioles during blood flow control. *Am. J. Physiol.* **263.** H1-H7.

Serras F, Damen P, Dictus W. G, Notenboom R. G and Van Der Biggelaar J. M. (1989). Communication compartments in the ectoderm of *Patella vulgata. Roux's Arch. Devlop. Biol.* **198.** 191-200.

Serras F, Fraser S and Chuong C-M. (1993). Assymetric patterns of gap junctional communication in developing chicken skin. *Development.* **119.** 85-96.

Sheardown S. A and Hooper M. L. (1992). A relationship between gap junction-mediated intercellular communication and the in vitro development capacity of murine embryonic stem cells. *Exp. Cell. Res.* **198.** 276-282.

Sheridan J. D, Finbow M. E and Pitts J. D. (1979), Metabolic interactions between animal cells through permeable intercellular junctions. *Exp. Cell. Res.* **123.** 111-117.

Sherman M. I. (1975). Differentiation of teratoma cell line PCC4.aza 1 *in vitro. In Teratomers and differentiation.* Sherman M. I and Stoler D. eds. New York Acadademic Press. pp 189-205.

Shimoyama Y, Nagafuchi A, Fujita S, Gotoh M, Takeichi M, Tsukita S and Hiohashi S. (1992). Cadherin dysfunction in a human cancer cell line: Possible involvement of loss of a-catenin expression in reduced cell-cell adhesiveness. *Cancer Res.* **52.** 5770-5774.

Short A. D, Bian J, Ghosh T. K, Waldron R. T, Rybak S. L and Gill D. L. (1993). Intracellular Ca++ pool content is linked to control of cell growth. *Proc. Natl. Acad. Sci.* **90**. 4986-4990.

Slack J. M, Darlington B. G, Heath J. K and Godsave S. F. (1987). Mesoderm induction in early Xenopus embryos by herapin-binding growth factors. *Nature*. **326**. 197-200.

Sleigh M. J. (1992). Differentiation and proliferation in mouse embryomal carcinoma cells. *Bioassays*. **14**. 769-775.

Sosinsky G. E. (1992). Image analysis of gap junction structures. *Electron. Microsc. Rev.* **5**. 59-76.

Sousa P. A, Valdimarsson G, Nicholson B. J and Kidder G. M. (1993). Connexin trafficking and the control of gap junction assembly in mouse preimplantation embryos. *Development*. **117**. 1355-1367.

Spray D. C, Fujita M, Saez J. C, Choi H, Watanabe T, Hertzberg E, Rosenberg L. C and Reid L. M. (1987). Proteoglycans and glycosaminoglycans induce gap junction synthesis and function in primary liver cultures. *J. Cell. Biol.* **105**. 541-551.

Spray D. C, Stern A. L and Bennett M. V. L. (1982). Comparison of sensitives to H+ and Ca++ ions. *Proc. Natl. Acad. Sci.* **79**. 441-445.

Spray D. C, Harris A. L and Bennet M. V. L. (1981). Equilbrium properties of voltage-dependent junctional conductance. *J. Gen. Physiol.* **77**. 75-94.

Spray D. C and Burt J. M. (1990). Structure-activity relations of the cardiac gap junction channel. *Am. J. Physiol.* **258**. 195-205

Spray D. C and Dermietzel R. (1995). X-Linked dominant Charcot-Marie-Tooth disease and other potential gap junction diseases of the nervous system. *Trends Neuro. Sci.* **18**. 256-262.

Stauffer K. A, Kumar N. M, Gilula N. B and Unwin N. (1991). Isolation and purification of gap junction channels. *J. Cell. Biol.* **115**. 141-150.

Stauffer P. L, Zhao H, Phelps K. L, Moss R. L, Star R.A and Muallem S. (1993). Gap junction communication modulates Ca++ oscillations and enzyme secretion in pancreatic acini. *J. Biol. Chem.* **268**. 19769-197789

Stein L. S, Stein D. W, Echols J and Burghardt R. C. (1993). Concomitant alterations of desosomes, adhesiveness, and diffusion through gap junction channels in a rat ovarian transformation model system. *Exp. Cell. Res.* 207. 19-32.

Steinberg T. H, Civitelli R, Geist S. T, Robertson A. J, Hick E, Veenstra R. D, Wang H-Z, Warlow P. M, Westphale E. M, Laing J. G and Beyer E.C. (1994). Connexin 43 and connexin 45 form gap junctions with different molecular permeabilities in osteoblastic cells. *EMBO J.* **13**. 744-750.

Stevens L. C. (1960). Embryonic potency of embryoid bodies from a transplantable testicular teratoma of the mouse. *Devel. Biol.* **2**. 285-297.

Stevens L. C. (1970). *Adv. Morphog.* **6**. 1-31.

Stewart T. A and Mintz B. (1981). Successive generations of mice produced from an established culture line of euploid teratocarcinoma cells. *Proc. Natl. Acad. Sci.* **78**. 6314-6318.

Stoker M. G. (1967). Transfer of growth inhibition between normal and virus-transformed cells: Autoradiographic studies using marked cells. *J. Cell Sci.* **2**. 293-304.

Stoker M. G. P, Shearer M and O'Neill C. (1966). Growth inhibition of polyoma-transformed cells by contact with normal fibroblasts. *J. Cell. Sci.* **1**. 297-310.

Strickland S and Mahdavi V. (1973). The induction of differentiation in teratocarcinoma stem cells by retenoic acid. *Cell.* **15**. 393-403.

Stutenkemper R, Geisse S, Schwartz H. J, Look J, Traub O, Nicholson B. J and Willecke K. (1992). The hepatocyte-specific phenotype of murine liver cells correlates with high expression of connexin32 and connexin26 but very low expression of connexin43. *Exp. Cell Res.* **201**. 43-54.

Sullivan R and Lo C. W. (1995). Expression of a connexin 43/b-Galactosidase fusion protein inhibits gap junctional communication in NIH-3T3 cells. *J. Cell. Biol.* **130**. 419-429.

Swenson K, Worms H, McNamee H and Paul D. (1990). Tyrosine phosphorylation of the gap junction protein connexin 43 is required for the pp60$^{v\text{-}src}$-induced inhibition of communication. *Cell Regulation*. **1**. 989-1002.

Swenson K. L, Jordan J. R, Beyer E. C and Paul D. L. (1989). Formation of gap junctions by expression of connexins in Xenopus oocyte pairs. *Cell.* **57**. 145-155.

Takeichi M. (1988). The cadherins: cell-cell adhesion molecules controlling animal morphogenesis. *Development*. 102. 639-655.

Takeichi M. (1990). Cadherins: A molecular family important in selective cell-cell adhesion. *Ann. Rev. Biochem.* **59**. 237-252.

Takeichi M. (1991). Cadherin cell adhesion receptors as a morphogenetic regulator. *Science.* **25**. 1451-1455.

Takeichi M. (1993). Cadherins in cancer: implications for invasion and metastasis. *Current opinion in biol.* **5**. 806-811.

Taylor S. S, Buechler J. A and Yonemoto W. (1990). cAMP-dependent protein kinase: framework for a diverse family of regulatory enzymes. *Ann. Rev. Biochem.* **59**. 971-1005.

Teranishi T, Negishi K and Kato S. (1983). Dopamine modulates S-potential amplitude and dye coupling between external horizontal cells in carp retina. *Nature*. **301**. 243-246.

Thiery J. P, Duband J. L and Delouvee A. (1982). Pathways and mechanism of avian trunk neural crest cell migration and localisation. *Dev. Biol.* **93**. 324-343.

Thomas L.A and Yamada K. M. (1992). Contact stimulation of cell migration. *J. Cell. Sci.* **103**. 1211-1214.

Thomsen G, Woolf T, Whitman M, Sokol S, Vaughan J, Vale W and Melton D. A. (1990). Activins are expressed early in Xenopus embryogenesis and can induce axial mesoderm and anterior structures. *Cell.* **63.** 485-493.

Tibbets T.T, Casper D. L. D, Phillips W. C and Goodenough D. A. (1990). Diffraction diagnosis of protein folding in gap junction connexons. *Biophys. J.* **57.** 1025-1036.

Tickle C. (1983). Positional signalling by retinoic acid in the developing chick wing. In *Limb development and regeneration*, part A, ed. Fallon J. F. pp89-98. New York: Alan R Liss, Inc.

Todaro G. J, Lazar G and Green H. (1965). The initiation of cell division in a contact inhibited mammalian cell line. *J. Cell. Comp. Physiol.* **66.** 325-334

Todaro G. J and Green H. J. (1963). *J. Cell Biol.* **17.** 299-310.

Tomasetto C, Neveu M. J, Daley J, Horan P. K and Sager R. (1993). Specificity of gap junction communication amoung human mammary cells and connexin transfectants in culture. *J. Cell. Biol.* **122.** 157-167.

Tomlinson A and Ready D. F. (1986). Sevenless: a cell specific homeotic mutation of the *Drosophila* eye. *Science.* **231.** 400-402.

Traub O, Look J, Dermietzel R, Brummer F, Hulser D and Willecke K. (1989). Comparative characterisation of the 21-kD and 26-kD gap junction proteins in murine liver and cultured hepatocyted. *J. Cell. Biol.* **108.** 1039-1051.

Traub O, Look J, Paul D and Willecke K. (1987). Cyclic adenosine monophosphate stimulates biosynthesis and phosphorylation of the 26kDa gap junction protein in cultured hepatocytes. *Eur. J. Cell Biol.* **43.** 48-54.

Troyanovsky S. M, Eshkind L. G, Troyanovsky R. B, Leube R. E and Franke W. W. (1993). Contributions of cytoplasmic domains of desmosomal cadherins to desmosome assembly and intermediate filament anchorage. *Cell.* **72.** 561-574

Tsakonas S. A, Matsuno K and Fortini M. E. (1995). Notch signalling. *Science.* **268.** 225-232.

Tsukita S, Tsukita S. Nagafuchi A and Yonemura S. (1992). Molecular linkage between cadherins and actin filaments in cell-cell adherins junctions. *Current Opinion in Cell Biol.* **4.** 834-839.

Turin L and Warner A. (1977). Carbon dioxide reversibly abolishes ionic communication between cells of early amphibian embryo. *Nature.* **270.** 56-58.

Turner C. E and Burridge K. (1991). Transmembrane molecular assembilies in cell-extracellular matrix interactions. *Curr. Opin. Cell Biol.* **3.** 849-853.

Unwin P. N. T and Ennis P. D. (1984). Two configurations of a channel-forming membrane protein. *Nature.* **307.** 609-613.

Veenstra R. D and Brink P. R. (1992). Patch-clamp analysis of gap junctional currents. In *Cell-Cell Interactions*. Stevensen B. R, Gallin W. J and Paul D. L, eds. IRL Press. pp 167-199.

Veenstra R. D. (1990). Voltage-dependent gating of gap junction channels in embryonic chick ventricular cell pairs. *Am. Physiol. Soc.* C662-C672.

Vleminckx K, Vakaet L, Mareel M, Fiers W and Roy F. V. (1991). Genetic manipulation of E-cadherin expression by epithelial tumour cells reveals an invasion suppressor role. *Cell.* **66.** 107-119.

Wakeford R. J. (1979). Cell contact and positional communiaction in *Hydra*. *J. Embryol. exp. Morph.* **54.** 171-183.

Walz D. A. (1992). Thrombospondin as a mediator of cancer cell adhesion in metastasis. *Cancer and metastasis.* **11.** 313-324.

Wang N, Butler J. P and Ingber D. E. (1993). Mechanotransduction across the cell surface and through the cytoskeleton. *Science.* **260.** 1124-1129.

Wardlaw A. C. (1992). Practical statistics for experimental biologists. Wiley. New York

Warner A. E, Guthrie S. C and Gilula N. B. (1984). Antibodies to gap junctional protein selectively disrupt junctional communication in the early amphibian embryo. *Nature* **311.** 127-131.

Warner A. E and Lawerence P. A. (1982). Permeability of gap junctions at the segmental border in insect epidermis. *Cell.* **28.** 243-252.

Watabe M, Nagafuchi A, Tsukita S and Takeichi M. (1994). Induction of polarized cell-cell association and retardation of growth by activation of the E-cadherin-catenin adhesion system in a dispersed carcinoma line. *J. Cell. Biol.* **127.** 247-256.

Werb Z, Tremble P. M, Behrendsten O, Crowely E and Damsky C. H. (1989). Signal transduction through the fibronectin receptor induces collagenase and stromelysin gene expression. *J. Cell Biol.* **109.** 877-889.

Wheelock M. J and Jensen P. J. (1992). Regulation of keratinocyte intercellular junction organization and epidermal morphogenesis by E-cadherin. *J. Cell. Biol.* **117.** 415-425.

White T. W, Bruzzone R, Goodenough D. A and Paul D. L. (1992) Mouse Cx50, a functional member of the connexin family of gap junction proteins is the lens fiber protein MP70. *Mol. Biol. Cell.* **3.** 711-720.

Wieser R. J, Schutz S, Tschank G, Thomas H, Dienes H-P and Oesch F.(1990). Isolation and characterisation of a 60-70-kD plasma membrane glycoprotein involved in the contact-dependent inhibition of growth. *J. Cell Biol.* **111.** 2681-2692.

Wilkie A. O, Kay M. G, Jones E. Y and Heath J. K. (1995). Functions of fibroblast growth factors and their receptors. *Current Biology.* **5.** 500-525.

Willecke K. R, Hennermann H, Dahl R, Jungbluth S and Heynkes R (1991). The diversity of connexin genes encoding gap junctional proteins. *Eur. J. Cell Biol.* **56.** 1-7.

Williams C. L, Hayes V. Y, Hummel A. M, Tarara J. E and Halsey T. J. (1993). Regulation of E-cadherin-mediated adhesion by muscarinic acetlycholine receptors in small cell lung carcinoma. *J. Cell. Biol*ogy. **121.** 634-654.

Wolpert L. (1989). Positional information revisited. *Development.* (Suppl.). 3-12.

Wolszon L. R, Rehder V, Kater S. B and Macagno E. R. (1994a). Calcium wave fronts that cross gap junctions may signal neuronal death during development. *J. Neuroscience.* **14.** 3437-3448.

Wolszon L. R, Gao W. Q, Passani B. M and Macagno E. R. (1994b). Growth cone collapse in vivo: Are inhibitory interactions mediated by gap junctions? *J. Neuroscience.* **14.** 999-1010.

Xu A, Meiners S and Schindler M. (1990). Immunological investigations of relatedness between plant and animal connexins. In *Parallels in cell-cell junctions in plants and animals*. Robards A. W, Jongsma H, Lucas W. J, Pitts J. D and Spray D, eds. Springer-Verlag. pp 171-183.

Yaholm A, Warmbrodt R. D, Laird D. W, Traub O, Revel J. P, Willecke K and Epel B. L. 1991. Maize mesocotyl plasmodesmata proteins cross-react with connexin gap junction protein antibodies. *Plant Cell.* **3.** 407-417.

Yamasaki H and Enomoto T. (1985). Role of intercellualr communication in BALB/c 3T3 cell transformation.*Carcinogenesis.* **9.** 179-194.

Yamasaki H and Katoh F. (1988). Further evidence for the involvement of gap junctional intercellular communication in induction and maintenance of transformed foci in BALB/c 3T3 cells. *Cancer Res.* **48.** 3490-3495.

Yamasaki H. (1991). Aberrant expression and function of gap junctions during carcinogenesis. *Environ. Health Perspect.* **93.** 191-197.

Yancey S. B, John S. A, Lal R, Austin B. J and Revel J. P. (1989). The 43-kD polypeptide of heart gap junctions: immunolocalisation (I), topology (II) and functional domains (III). *J. Cell. Biol.* **108.** 2241-2254.

Yeager M and Nicholson B. J. (1996). Structure of gap junction intercellular channels. *Curr. Opin. Struc. Biol.* **6.** 183-192.

Young D, Waitches G, Birchmeir C, Fasano O and Wigler M. Isolation and characterisation of a new cellular oncogene encoding a protein with multiple potential transmembrane domains. *Cell.* **45.** 711-719.

Yurochko A. D, Liu D. Y, Eierman D and Haskill S. (1992). Integrins as a primary signal transduction molecule regulation monocyte immediate early gene induction. *Proc. Natl. Acad. Sci.* **89.** 9034-9038.

Yuste R, Peinado A and Katz L. C. (1992). A system of neuronal domains in developing neocortex. *Science.* **257.** 665-668.

Zachary I and Rozengurt E. (1992). Focal adhesion kinase (p125fak): A point of convergence in the action of neuropeptides, integrins and oncogenes. *Cell.* **71.** 891-894.

Zampighi G. A, Bosch E and Ramon F. (1988). Some observations about the cellular mechanisms involved in gap junction regulation. In *Gap Junctions*, ed. Hertzberg E. L and Johnson R. G. pp 151-164. Alan Liss Inc. New York.

Zecca M Basler K and Struhl G. (1995). Sequential organising activities of engrailed, hedgehog and decapentaplegic in the drosophila wing. *Development* **121**. 2265-2278.

Zetter B. R. (1993). Adhesion molecules in tumour metastasis. *Cancer Biology*. **4**. 219-229

Zhu D, Kidder G. M, Caveney S and Naus C. C. G. (1992). Growth retardation in glioma cells cocultured with cells overexpressing a gap junction protein. *Proc. Natl. Acad. Sci.* **89**. 10218-10221.

Zhu X and Assoian R. K. (1995). Integrin-dependent activation of MAP kinase: a link to shape-dependent cell proliferation. *Mol. Biol. Cell*. **6**. 273-282.

GLASGOW UNIVERSITY LIBRARY