

N-glycosylated proteins interfere with the first cellular migrations in early chick embryo

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ABSTRACT Cell adhesion and migration properties which are known to play a crucial role in developmental events seem to be modulated by variations in glycosylation of glycoproteins. In the chick embryo, the extracellular matrix (ECM) appears as a loose meshwork of fibrillar material in the space between the epiblast and the hypoblast shortly before the first major cell migrations start. Chick embryos treated with tunicamycin (TN), a specific inhibitor of N-linked glycosylation of proteins, show little or no ECM, diminished cell adhesion and a dramatic alteration in the architecture of the epiblast and of the hypoblast. The first major cell migrations which signal the onset of PS and gastrula formation are inhibited irreversibly in these embryos. Tunicamycin induces a substantial change in the labeling pattern with change in mobility of some polypeptides and with the induction or marked accentuation of multiple charged species (isoforms) of polypeptides different from these already present in the control blastoderm. The N-linked glycosylation of protein(s) that are synthesized during the interaction of the epiblast and of the hypoblast seem to play a critical role in cell adhesion and in the morphogenetic movements of gastrulation in the early chick embryo.

KEY WORDS: *cellular migration, adhesion, extracellular matrix, N-glycosylation, chick embryo*

Glycoproteins are integral components of membranes and of the extracellular matrix (ECM) and are involved in several aspects of cellular behavior including growth, response to signal molecules, migration, adhesion, interaction with, and recognition of, other cells (Manasek, 1975; England, 1982; Sanders, 1983; Sharon, 1984; Thiery, 1984; West, 1986; Ruoslahti and Pierschbacher, 1987). Cellular behavior may be modulated by variations in glycosylation of glycoproteins and/or glycolipids. The availability of specific inhibitors acting at different stages in the glycosylation process has permitted the synthesis of protein without oligosaccharide moieties or protein with altered oligosaccharide structures. The inhibitors of glycosylation inhibit embryonic development and differentiation (Olden *et al.*, 1985; West, 1986).

Tunicamycin is a structural analogue of UDP-2-acetamide-2-deoxyglucose and inhibits selectively the initial step in the N-glycosylation of glycoproteins (Takatsuki *et al.*, 1971, 1975; Tkacz and Lampen, 1975; Struck and Lennarz, 1977). It has been used as a specific probe for the function of N-glycosidically linked oligosaccharide moieties. This inhibitor of N-glycosylation causes a dramatic alteration in cell shape and surface morphology in chick embryo fibroblasts (Duksin and Bornstein, 1977; Pratt *et al.*, 1979), inhibits migration of corneal epithelial sheets (Gipson *et al.*, 1984), and arrests the development of sea urchin (Heifetz and Lennarz, 1979; Ruoslahti and Pierschbacher, 1987) and amphibian embryos (Romanovsky and Nosek, 1980) at the gastrula stage and

mammalian embryos at the stage of compaction and blastocyst formation (Surani, 1979; Atienza-Samols *et al.*, 1980; Iwakura and Nozaki, 1985; Olden *et al.*, 1985).

The first morphogenetic events in the early chick embryo depend on processes involving cellular adhesion, interaction and migration. The primary hypoblast is formed partly by polyingression of cells from the upper layer (epiblast) and partly by spreading of cells from the posterior marginal zone (PMZ) of the epiblast. The hypoblast interacts with the epiblast, and the PMZ component of the hypoblast induces the first extensive cellular migrations and formation of the primitive streak (PS) in the epiblast (Eyal-Giladi and Khaner, 1989; Khaner and Eyal-Giladi, 1989). The PS is the site through which cells migrate to form mesoderm and definitive endoderm. The mesodermal cells interact with the overlying ectodermal cells which are induced to form the neural plate (Vakaet, 1970; Eyal-Giladi, 1984; Gurdon, 1987). The oligosaccharide chains of the N-linked glycoproteins are believed to be implicated in a variety of recognition phenomena ranging from cellular aggregation to participation in embryonic development. We have used tunicamycin to study the role of N-linked oligosaccharides of glycoproteins on the first cellular migrations and inductions in the early chick embryo.

Abbreviations used in this paper: ECM, extracellular matrix; PMZ, posterior marginal zone; PS, primitive streak; TN, tunicamycin.

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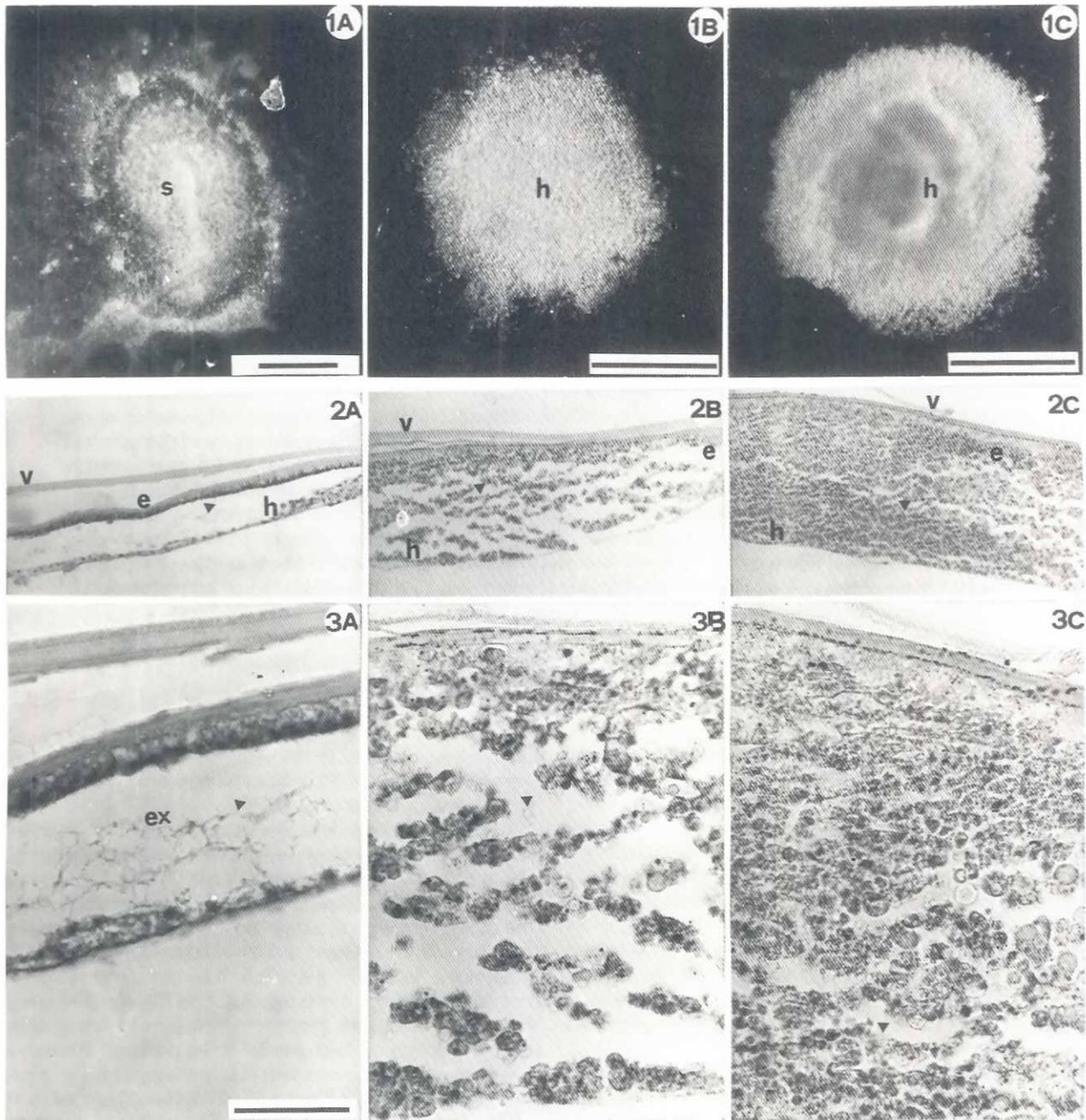
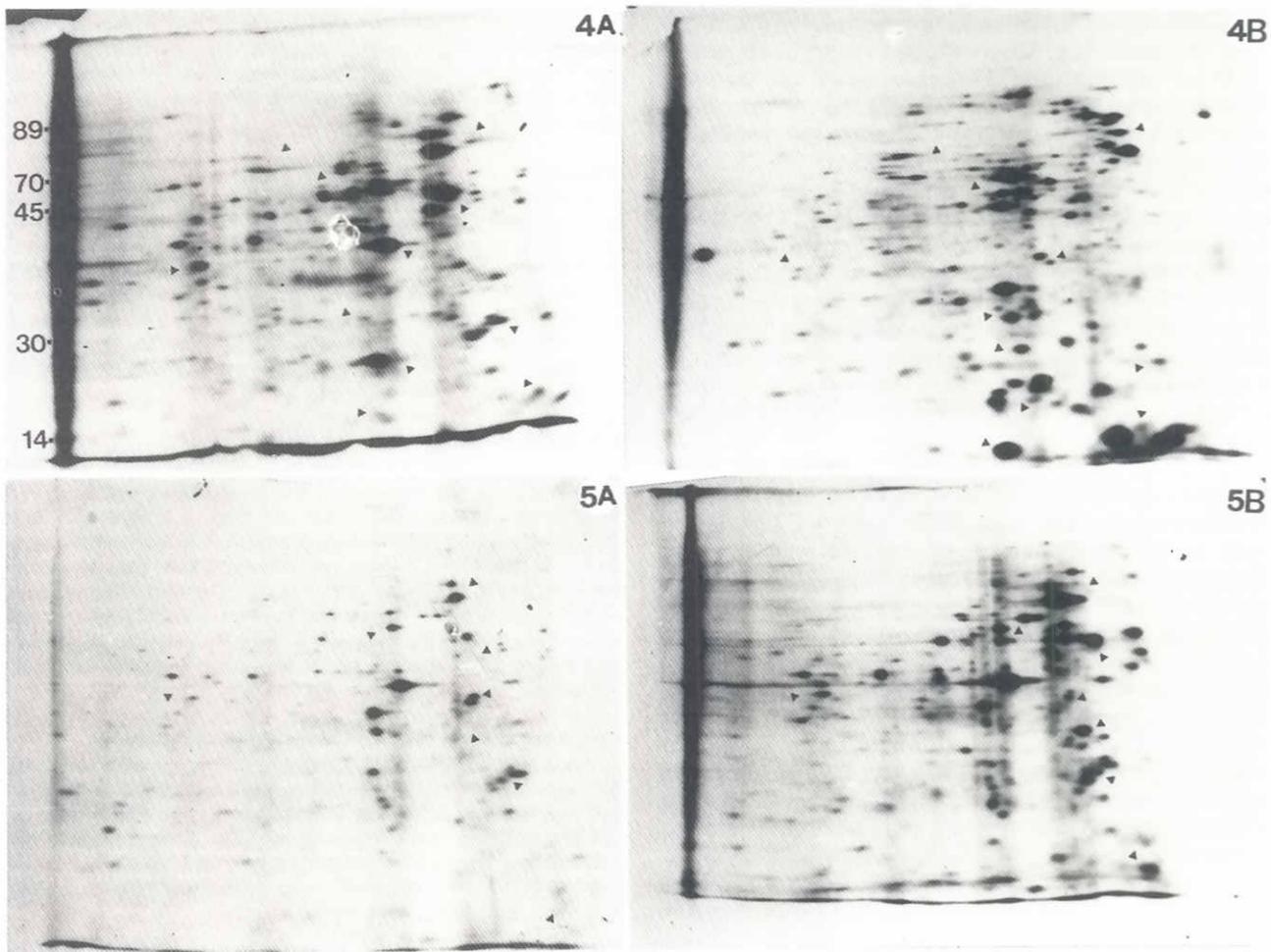


Fig. 1. Effect of tunicamycin on induction of embryonic axis in chick embryo. Embryos at stage 10 (A, B) and stage 13 (C) were cultured in plain egg albumen (A), in egg albumen containing 5.9×10^{-6} M tunicamycin for 2 h then transferred to plain egg albumen (B, C) and photographed 23 h after beginning of culture. h, hypoblast; s, primitive streak. Bar, 1 mm.

Figs. 2 and 3. Transverse sections ($5 \mu\text{m}$) through the center of embryo presented in Fig. 1B (2B, 3B) and the embryo presented in Fig. 1C (2C, 3C). Non-treated embryo at stage 13 is presented for comparison (2A, 3A). Sections are stained with Alcian blue stain. Arrowhead shows location of lower magnification sections (Fig. 2) presented under higher magnification (Fig. 3). e, epiblast; ex, extracellular matrix; h, hypoblast; v, vitelline membrane. Bar, $50 \mu\text{m}$.

The formation of the PS is the first major morphogenetic event which signals the gastrula formation in the early chick embryo. Induction of the PS is a process involving cell adhesion, interaction and cellular migration. These processes appear to be modulated by

the expression of adhesion molecules or by variation in glycosylation of components of the ECM (Manasek, 1975; England, 1982; Edelman 1984; Sharon, 1984; Thiery, 1984; Olden *et al.*, 1985; West, 1986; Harrison *et al.*, 1988). The first cellular migrations for



Figs. 4 and 5. Two-dimensional gel pattern of polypeptides of chick embryos at stages 10 and 13 after treatment with tunicamycin. Embryos at stages X (**4A, B**) and thirteen (**5A, B**) cultured in chick Ringer solution in the absence (A) and in the presence (B) of tunicamycin (5.9×10^{-6} M) at 37°C for 8 h (control) or 18 h (experimental) and labeled with ^{35}S -methionine the last 4 h in culture. Samples were analyzed by isoelectric focusing gels and subjected to two-dimensional electrophoresis. Isoelectric focusing is from left (pH 7.2) to right (pH 4.4). Molecular mass (daltons $\times 10^{-3}$) of polypeptides identified was determined according to electrophoretic migration of standards (Rainbow markers, Amersam). Arrowheads indicate changes in major polypeptides.

formation of the PS are inhibited when chick embryos at stages 10 (Fig. 1B) and thirteen (Fig. 1C) are exposed to tunicamycin for 2 h. Embryos cultured in the absence of tunicamycin form the embryonic axis in parallel cultures (Fig. 1A). The minimal concentration of tunicamycin treatment that produces the effects described above is 2.3×10^{-6} during a 2 h culture. In cultures continuously in tunicamycin, the embryos collapse and cannot be lifted intact from the vitelline membrane raft.

Sections through the embryo which started TN treatment at stage 10 presented in Fig 1B (Figs. 2B, 3B) and through the embryo which started TN treatment at stage thirteen in Fig. 1C (Figs. 2C, 3C) do not show EC material after staining with Alcian blue, which reveals the presence of glycosaminoglycans. The embryo has lost its organization in that the cells of the epiblast and the hypoblast cannot be recognized as two separate layers but appear as a loose association of cells. All of the proteins that have been implicated in

cell-cell adhesion are glycosylated, and there are several lines of evidence that suggest a role(s) for oligosaccharide moieties in the process of cell-cell adhesion. Section through an embryo at stage thirteen which is presented for comparison (Figs. 2A, 3A) (Zagris *et al.*, 1989) shows the epiblast, the hypoblast and the ECM, which appears as an intricate network in the blastocoel after staining with Alcian blue.

Embryos at stages 10 and thirteen were cultured in the absence (control) or in the presence of TN (5.9×10^{-6} M) for 8 h or 18 h, respectively, and labeled with ^{35}S -methionine the last 4 hr of the culture period. The polypeptide synthesis, analyzed by two-dimensional gel electrophoresis, shows several qualitative and quantitative changes between control and TN-treated embryos. Embryos which started TN treatment at stage 10 expressed many isoforms in polypeptides at apparent molecular mass (Mr) of 75/90 Kd complex, 45/70 Kd complex, 35, 30, 25, and 14 Kd. Moreover, they

showed great increases in synthesis of polypeptides at 35, 30, 20, and 14 Kd, decreases in synthesis of polypeptides at 40, and 35 Kd, and the new appearance of polypeptides at 25, 20, and 14 Kd after TN-treatment as compared to control (Fig. 4B, compare to 4A). Embryos which started TN treatment at stage 13 showed an increase in synthesis of polypeptides at 80, 60, 35, 30, 20 and 14 Kd, decrease in synthesis of polypeptides at about 40 Kd and appearance of polypeptides at 65 and 60 Kd after TN-treatment as compared to control (Fig. 5B, compare to 5A). Substantial changes in the labeling pattern with changes in mobility of some polypeptides towards lower Mr and with the induction or marked accentuation of multiple charged species (isoforms) of polypeptides different from these already present in the control embryos were observed in TN-treated embryos (Figs. 4, 5). Inhibition of N-glycosylation interferes with the normal production of ECM and the first major cell migrations are inhibited.

It is known that during the initial cleavages, sea urchin and mouse embryos are relatively resistant to TN although treatment with the antimetabolite from early stages can have serious consequences during subsequent development (Schneider *et al.*, 1978; Heifetz and Lennarz, 1979; Atienza-Samols, 1980). This is in concert with our results which show that the treatment of chick embryo at stage 10 (homologous to the morula stage) with TN inhibits the morphogenetic movements of gastrulation that are induced in embryos during stage 13 (homologous to blastula). Thus, it seems that the synthesis of glycoproteins, specifically those containing asparagine-linked units is required for the normal production of ECM and for the migration of cells to form the PS and for gastrulation. It is known that removal of N-linked oligosaccharides impairs neural induction in amphibians (Gualandris-Parisot *et al.*, 1991). This effect could result from faulty translocation, secretion, instability and/or inactivity of glycoproteins required for these processes, or the asparagine-linked carbohydrate unit itself may be involved directly in cell-cell recognition and adhesion.

Experimental Procedures

Embryo culture

Fertilized chick eggs were used. Embryos at stages 10 and 13 (Eyal-Giladi and Kochav, 1976) were removed from the eggs and cleaned free of the vitelline membrane and any adhering yolk in chick Ringer solution. Support «rafts» were made of vitelline membrane (New, 1955) and embryos were flattened with the epiblast side against the surface of a raft. Embryos were placed on plain Ringer solution (control) or on Ringer solution containing tunicamycin (Calbiochem-stock solution 1 mg/ml dimethylsulfoxide) to give final concentrations from 2.3×10^{-6} to 11.9×10^{-6} M and were incubated at 37°C for various times from 2 h to 17 h. After treatment with tunicamycin, the underside of each raft was washed carefully and culture was continued for at least an additional 20 h on plain egg albumen.

Treated and control embryos were observed and photographed at various time intervals. Fifteen embryos, usually ten experimental and five control, were cultured per stage per experiment. A total of 60 embryos were used at each experimental stage in different experiments. Sterile procedures were employed in all embryo cultures.

Differential staining of extracellular material

Embryos were fixed for 4-16 h in Gendre fluid (95% ethyl alcohol saturated with picric acid; concentrated formalin; glacial acetic acid, 8.0:1.5:0.5) fixative known to preserve the ECM material (Humason, 1972; Webster *et al.*, 1983). Embryos were washed in 80% ethanol, were dehydrated through graded ethanol with two 5 min changes and routinely prepared for paraffin sectioning. Serial sections (5µm) were deparaffinized and rehydrated sequentially in graded alcohols down to H₂O. The Alcian blue

8GX (Sigma, St. Louis, Mo) – pH 2.5 staining technique was used to stain the ECM. Alcian blue reveals the presence of glycosaminoglycans which give a turquoise color (Humason, 1972). Nuclear fast red (Sigma) was used as a counter stain. Stained sections were dehydrated and mounted following conventional procedures.

Labeling

Embryos at stages 10 and 13 were placed in plain Ringer solution in the absence (control) or presence of TN at a final concentration of 5.9×10^{-6} M for 8 h or 18 h, respectively, at 37°C. The rafts were transferred to fresh media containing the previous concentrations of TN and $295 \mu\text{Ci L}^{-1}$ [³⁵S]-methionine (1,094Ci/mmol, New England Nuclear -009T) per milliliter for 4 h at 37°C. Labeling was terminated by chilling the embryos on ice. Embryos were washed in ice-cold Ringer and were detached from the vitelline membrane rafts with fine dissecting needles (Zagris and Matthopoulos, 1985, 1986). Sterile procedures were employed.

Sample preparation

Embryos at stages 10 and 13 (3 embryos per group) were labeled as described in the previous section and homogenized separately in 30 µl of RSB buffer (10 mM Tris-HCl, 10 mM NaCl, 1.5 mM MgCl₂, pH 7.4) containing 0.5% Zwittergent 3-12 detergent (Calbiochem-Behring, La Jolla, Calif.). To determine [³⁵S]-methionine incorporation into newly synthesized proteins, 2 µl of a homogenate was spotted onto a Whatman GF/C filter. Filters were placed in a series of trichloroacetic acid and ethanol-ether washes (Zagris and Matthopoulos, 1985). Radioactivity was measured by scintillation counting in 10 ml toluene containing 0.5% PPO and 0.03% POPOP.

Protein analysis by two-dimensional gel electrophoresis

The species of labeled polypeptides were analyzed by two-dimensional gel electrophoresis using isoelectric focusing in the first dimension (O'Farrell, 1975) and polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970) in the second dimension, and fluorography. The gels were loaded with equal incorporated counts of radioactivity (approximately 90,000 cpm). These methods have been described more extensively elsewhere (Zagris and Matthopoulos 1985, 1986, 1987).

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References

- ATIENZA-SAMOLS, S.B., PINE, P.R. and SHERMAN, N. (1980). Effects of tunicamycin upon glycoprotein synthesis and development of early mouse embryos. *Dev. Biol.* 79: 19-32.
- DUKSIN, D. and BORNSTEIN, P. (1977). Changes in surface properties of normal and transformed cells caused by tunicamycin, an inhibitor of protein glycosylation. *Proc. Natl. Acad. Sci. USA* 74: 3433-3437.
- EDELMAN, G.M. (1984). Expression of cell adhesion molecules during embryogenesis and regeneration. *Exp. Cell Res.* 161: 1-16.
- ENGLAND, M.A. (1982). Interactions at basement membranes. In *Functional Integration of Cells in Animal Tissues* (Eds. J.D. Pitts and M.E. Finbow). Cambridge University Press.
- EYAL-GILADI, H. (1984). The gradual establishment of cell commitments during the early stages of chick development. *Cell Differ.* 14: 245-255.
- EYAL-GILADI, H. and KHANER, O. (1989). The chick's marginal zone and primitive streak formation. II. Quantification of the marginal zone's potencies-temporal and spatial aspects. *Dev. Biol.* 134: 215-221.
- EYAL-GILADI, H. and KOCHAV, S. (1976). From cleavage to primitive streak formation: A complementary normal table and a new look at the first stages of the development of the chick. I. General morphology. *Dev. Biol.* 49: 321-337.
- GIPSON, I.K., KIORPES, T.C. and BRENNAN, S.J. (1984). Epithelial sheet movement: effects of tunicamycin on migration and glycoprotein synthesis. *Dev. Biol.* 101: 212-220.

- GUALANDRIS-PARISOT, L., MAUREL, P., TEISSIE, J. and DUPRAT, A.M. (1991). Removal of N-linked oligosaccharides of presumptive ectoderm impairs neural induction in *Pleurodeles waltl*. *Mech. Dev.* 33: 201-214.
- GURDON, J.B. (1987). Embryonic induction molecular prospects. *Development* 99: 285-306.
- HARRISSON, F., ANDRIES, L. and VAKAET, L. (1988). The chicken blastoderm: current view of cell biological events guiding intercellular communication. *Cell Differ.* 22: 83-106.
- HEIFETZ, A. and LENNARZ, W.J. (1979). Biosynthesis of N-glycosidically linked glycoproteins during gastrulation of sea urchin embryos. *J. Biol. Chem.* 254: 6119-6127.
- HUMASON, G.L. (1972). *Animal Tissue Techniques*. Freeman and Co., San Francisco.
- IWAKURA, Y. and NOZAKI, M. (1985). Effects of tunicamycin on preimplantation mouse embryos: prevention of molecular differentiation during blastocyst formation. *Dev. Biol.* 112: 135-144.
- KHANER, O. and EYAL-GILADI, H. (1989). The chick's marginal zone and primitive streak formation. I. Coordinative effect of induction and inhibition. *Dev. Biol.* 134: 206-214.
- LAEMMLI, V.K. (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* 227: 680-685.
- MANASEK, F.J. (1975). The extracellular matrix: a dynamic component of the developing embryo. In *Current Topics in Developmental Biology* (Eds. A.A. Moscona and A. Monroy). Vol. 10. Academic Press, New York, pp. 35-102.
- NEW, D.A.T. (1955). A new technique for the cultivation of the chick embryo *in vitro*. *J. Embryol. Exp. Morphol.* 3: 326-331.
- O'FARRELL, P.H. (1975). High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250: 4007-4021.
- OLDEN, K., BERNARD, B.A., HUMPHRIES, M.J., YEO, T-K., YEO, K-T., WHITE, S.L., NEWTON, S.A., BAUER, H.C. and PARENT, J.B. (1985). Function of glycoprotein glycans. *Trends Biochem. Sci.* 10: 78-82.
- PRATT, R.M., YAMADA, K.M., OLDEN, K., OHANIAN, S.H. and HASCALL, V.C. (1979). Tunicamycin-induced alterations in the synthesis of sulfated proteoglycans and cell surface morphology in the chick embryo fibroblast. *Exp. Cell Res.* 118: 245-252.
- ROMANOVSKY, A. and NOSEK, J. (1980). The effect of tunicamycin and 2-deoxy-D-glucose on the development of *Xenopus laevis* embryos. *Roux Arch. Dev. Biol.* 189: 81-82.
- RUOSLAHTI, E. and PIERSCHBACHER, M.D. (1987). New perspectives in cell adhesion: RGD and integrins. *Science* 238: 491-497.
- SANDERS, E.J. (1983). Recent progress towards understanding the roles of the basement membrane in development. *Can. J. Biochem. Cell Biol.* 61: 949-956.
- SCHNEIDER, E.G., NGUYEN, H.T. and LENNARZ, W.J. (1978). The effect of tunicamycin, an inhibitor of protein glycosylation, on embryonic development in sea urchin. *J. Biol. Chem.* 253: 2348-2355.
- SHARON, N. (1984). Glycoproteins. *Trends Biochem. Sci.* 9: 198-202.
- STRUCK, D.K. and LENNARZ, W.J. (1977). Evidence for the participation of saccharide lipids in the synthesis of the oligosaccharide chain of ovalbumin. *J. Biol. Chem.* 252: 1007-1013.
- SURANI, M.A.H. (1979). Glycoprotein synthesis and inhibition of glycosylation by tunicamycin in preimplantation mouse embryos: Compaction and trophoblast adhesion. *Cell* 18: 217-227.
- TAKATSUKI, A., ARIMA, K.A. and TAMURA, G. (1971). Tunicamycin, a new antibiotic. I. Isolation and characterization of tunicamycin. *J. Antibiot.* 24: 215-223.
- TAKATSUKI, A., KOHNO, K. and TAMURA, G. (1975). Inhibition of biosynthesis of polyisoprenyl sugars in chick embryo microsomes by tunicamycin. *Agr. Biol. Chem.* 39: 2089-2091.
- THIERY, J.P. (1984). Mechanisms of cell migration in the vertebrate embryo. *Cell Differ.* 15: 1-15.
- TKACZ, J.S. and LAMPEN, J.O. (1975). Tunicamycin inhibition of polyisoprenyl N-acetylglucosamine pyrophosphate formation in calf-liver microsomes. *Biochem. Biophys. Res. Commun.* 65: 248-257.
- VAKAET, L. (1970). Cinematographic investigations of gastrulation in the chick blastoderm. *Arch. Biol. (Liège)* 81: 387-426.
- WEBSTER, Jr., E.H., SILVER, A.F. and GONSALVES, N.I. (1983). Histochemical analysis of extracellular matrix material in embryonic mouse lens morphogenesis. *Dev. Biol.* 100: 147-157.
- WEST, C.M. (1986). Current ideas on the significance of protein glycosylation. *Mol. Cell. Biochem.* 72: 3-20.
- ZAGRIS, N. and MATTHOPOULOS, D. (1985). Patterns of protein synthesis in chick blastula: a comparison of the component areas of the epiblast and the primary hypoblast. *Dev. Genet.* 5: 209-217.
- ZAGRIS, N. and MATTHOPOULOS, D. (1986). Differential heat shock gene expression in chick blastula. *Roux Arch. Dev. Biol.* 195: 403-407.
- ZAGRIS, N., MATTHOPOULOS, D. (1987). Induction of gastrulation in chick embryo. *Dev. Genet.* 8: 83-89.
- ZAGRIS, N., PANAGOPOULOU, M. and ANASTASSOPOULOS, V. (1989). Extracellular matrix organized in embryonic cavities during induction of the embryonic axis in chick embryo. *Cell Biol. Int. Rep.* 13: 833-843.

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