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Petr Dvorak, Evelyne Campion, Jacques Fléchon, Ales Hampl, Jean Renard. Modulation of protein synthesis in rabbit inner cell mass-derived cells by FGF-2. *Reproduction Nutrition Development*, 2001, 41 (3), pp.197-205. 10.1051/rnd:2001123 . hal-00900349

HAL Id: hal-00900349

<https://hal.science/hal-00900349>

Submitted on 11 May 2020

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Original article

Modulation of protein synthesis in rabbit inner cell mass-derived cells by FGF-2

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(Received 16 January 2001; accepted 2 April 2001)

Abstract — Gastrulation is a critical step in vertebrate development, that depends on synergistic effects of several signalling molecules, including fibroblast growth factor-2 (FGF-2). To follow this phenomenon *in vitro* we isolated rabbit inner cell masses (ICMs) at embryonic day 4 and we exposed ICM-derived cells to FGF-2. Then, we analysed the quantitative differences in rates of protein synthesis from day 3 to day 5 of culture by two-dimensional (2D) gel electrophoresis. Here we show that both up- and down-regulation of protein synthesis took place in ICM-derived cells upon their exposure to FGF-2. The effect of FGF-2 was most pronounced at day 4 of culture, when the changes were very much in favour of a set of down-regulated proteins. To test the significance of this period of time for FGF-2-mediated regulation of protein synthesis, cells were grown without FGF-2 and then they were pulse-treated with FGF-2 at the end of day 4. When compared to the continuous culture with FGF-2, the FGF-2 pulse resulted in a quite indistinguishable pattern of up- and down-regulated proteins. Thus, the readiness of ICM-derived cells to accept and respond to the FGF-2 signals may be of limited duration.

rabbit / inner cell mass / protein synthesis / FGF-2 / two-dimensional gel electrophoresis

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1. INTRODUCTION

Similarly to other mammals, the ICM of the rabbit embryo is the source of all foetal tissues. Cells within the large, flat rabbit ICM are first allocated to primitive ectoderm and primitive endoderm. Then, possibly under the control of growth factors, cells originating within primitive ectoderm give rise to mesoderm. Mesoderm diversifies into three populations – axial mesoderm, that contributes to organizing the basic body axis; somitic mesoderm, which gives rise to bone, muscle and dermis; and lateral mesoderm, which forms the body cavities and contributes to several organs. In contrast to the mouse, rabbit gastrulation occurs before implantation [12]. This makes the rabbit embryo a well accessible model for analysing the expression of genes that are specific to gastrulation. However, the significance of the data obtained *in vivo* is low due to the large asynchrony among embryos in multiparous rabbit females. Here, we overcame this limitation by using ICMs isolated from blastocysts that were synchronised in culture since the morula stage.

As mentioned above, growth factors may play crucial roles during the initial stages of gastrulation. One of them, FGF-2, has been involved in the differentiation of mesoderm in amphibian development [7, 21, 22, 23], and increasing evidence suggests that it also plays a similar role in mammals, including in the rabbit [5, 9, 11]. The process of mesoderm induction is displayed by the expression of specific mRNAs [18, 24] and proteins [5, 13, 28]. Besides analysing specific gene products directly involved in mesoderm induction or accompanying morphogenetic movement during that stage, characterising the overall changes in protein synthesis should also be emphasized. One useful approach is the construction of protein databases using high-resolution, 2D gel electrophoreses followed by computer-assisted analyses. Several mammalian databases, with the exception of the rabbit species, already provide information

regarding the regulation of protein synthesis in preimplantation embryos. Such databases were published for one-cell stage to blastocyst stage mouse embryos [3, 14, 16, 19]. Others focus on the quantitative analysis of proteins synthesized by 6.5–7.5 day mouse embryos with respect to differentiation of ectoderm, endoderm and mesoderm [15], proteins specific to major organogenesis in the mouse from day 7.5 to day 9.0 of development [25] or the developing heart of a 9.5 day mouse embryo [26]. Concerning potential morphogens, of all the growth factors that are known to affect embryonic development, only the effects of insulin growth factor on protein synthesis in eight-cell mouse embryos and blastocysts [20] and porcine embryonic discs [8], and the effect of TGFs on total protein synthesis in mouse blastocysts [1] were investigated.

Taking advantage of our previously established model of *in vitro* mesoderm induction in the rabbit [5], we analysed protein synthesis in ICM-derived cells that were exposed to a candidate morphogen FGF-2 during the period when the initial steps of rabbit gastrulation occur *in vivo*. We show that exogenous FGF-2 can stimulate and/or inhibit protein synthesis in rabbit embryonic cells in a pattern that may resemble the developmental specification of originally totipotent cells.

2. MATERIALS AND METHODS

2.1. Isolation and culture of ICMs

Rabbit morulas were collected at day 3 p.c. by oviduct flushing and were cultured individually overnight to the blastocyst stage in Dulbecco's modified Eagle medium (DMEM; Gibco/BRL, Gaithersburg, MD) supplemented with 20% foetal calf serum, 2 mM glutamine, 0.1 mM non-essential amino acids, 0.05 mM β -mercaptoethanol, 100 IU·mL⁻¹ penicillin and 0.1 mg·mL⁻¹ streptomycin. Then, only well developed blastocysts were subjected to immunosurgery

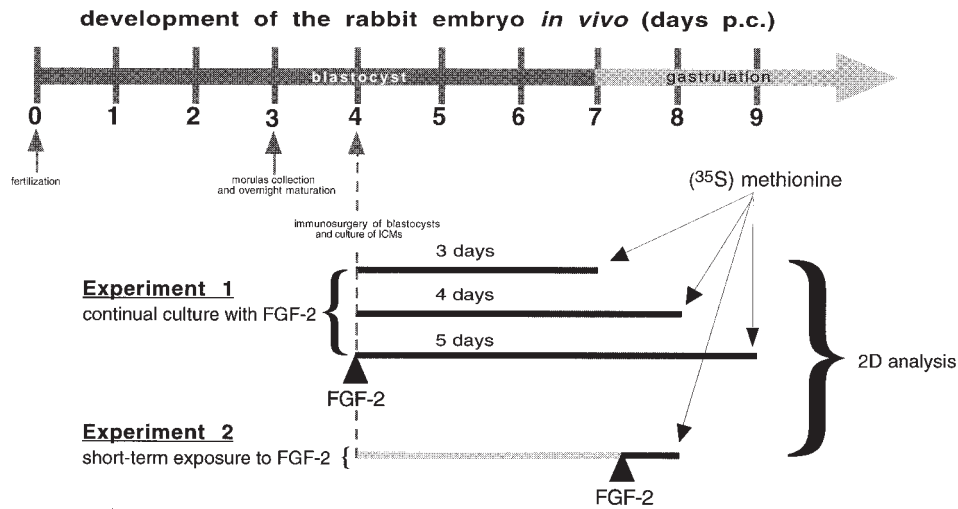


Figure 1. Experimental design of 2D gel analysis. Rabbit ICM-derived cells were exposed to the two concentrations of FGF-2 either from immunosurgery of blastocysts to day 5 of culture (Experiment 1) or only for 16 hours beginning from hour 80 of culture (Experiment 2).

in order to isolate ICMs. Immunosurgery was performed essentially according to the method of Babinet and Bordenave [2] using goat anti-rabbit whole serum (Sigma, St. Louis, MO) and guinea pig complement (Sigma). After isolation, four ICMs/well were placed on gelatin-coated 4-well dishes (Nunc, Roskilde, Denmark) and cultured in DMEM with 1 and/or 5 ng·mL⁻¹ FGF-2 (Sigma) until labelling and 2D analysis (Fig. 1; Experiment 1). For short-term exposure to FGF-2, ICMs were cultured for 80 hours without FGF-2, then exposed to 1 and/or 5 ng·mL⁻¹ FGF-2 for 16 hours and subjected to analysis (Fig. 1; Experiment 2). For both experiments, control ICMs were cultured in medium without FGF-2.

2.2. Radiolabelling and 2D gel electrophoresis

ICM-derived cells grown in the presence or absence of FGF-2 were scraped off the dish and washed twice with phosphate-buffered saline, pH 7.2 (PBS; Ca²⁺-Mg²⁺-free). Then cells were suspended in

M2 medium containing 0.4% polyvinylpyrrolidone (Sigma) and labelled with 1.5 mCi·mL⁻¹ [³⁵S]-methionine (specific activity 1500 Ci·mmol⁻¹; Amersham, Buckinghamshire, England) for 2 hours. After labelling, cells were extensively washed with cold PBS, proteins were extracted in lysis buffer (9.5 M urea, 5% β-mercaptoethanol, 0.5% NP-40, 0.5% SDS, 2.5% glycerol, 0.2% ampholines) and incorporation of [³⁵S]-methionine was determined by TCA precipitation followed by scintillation counting. Labelled proteins were then separated by non-equilibrium pH gradient electrophoresis [17] using pH 3–10 gradient ampholines (NEPHGE; 4% acrylamide, 9.5 M urea, 2% NP-40, 2% acrylamide, 9.5 M urea, 2% NP-40, 2% ampholines) an 11% SDS-PAGE in the second dimension. Between 0.5 and 1 × 10⁶ cpm were loaded onto each gel. The gels were then fixed, soaked in Amplify (Amersham), dried, and exposed to Fuji films at -70 °C. For each treatment, several experimental replicates were done and two representative gels showing the largest number of high-quality spots were selected for detailed analysis.

2.3. Image analysis

The gel autoradiograms were scanned (Ultrosan, Pharmacia, Uppsala, Sweden) and processed using Image Master System (Pharmacia) as follows. First, the migration coordinates were identified for all spots. Using these coordinates, the standard protein maps were created representing the set of protein spots that are detectable under two experimental conditions (FGF-2-treated and FGF-2-nontreated) and for each culture timing. Then the optical densities (OD) were determined for each spot and the relative synthesis rate of individual proteins for each treatment was quantified as a fold change of OD between the spot being evaluated (FGF-2-treated) and the identically migrating spot in the control gel (FGF-2-nontreated).

3. RESULTS

3.1. FGF-2 induces changes in the accumulation of proteins synthesized in rabbit ICM-derived cells

First, we determined the total number of protein spots that is typical for each culture condition (FGF-2 concentrations and culture timing). Routinely, we were able to identify an average of 200 proteins of which at least 90% were common to both experi-

mental conditions (FGF-2-treated and control nontreated). Only spots, the density of which increased and/or decreased at least two-fold when compared to nontreated controls, were classified as changing.

Upon continuous treatment with FGF-2 in both concentrations, about 11% of proteins up-regulated their accumulation at day 3. Twenty-four hours later, however, the percentage of proteins that increased their accumulation was much lower, 3.1 and 4.2% in cultures supplemented with 1 and 5 ng·mL⁻¹ FGF-2, respectively. Then, at day 5 of culture the level of up-regulation increased back to 9.2 and 10% in 1 and 5 ng·mL⁻¹ of FGF-2, respectively (Fig. 2; INCREASE).

In fact, in a certain sense the opposite is true for proteins that down-regulate their accumulation (Fig. 2; DECREASE). On the one hand, the most significant down-regulating effect of FGF-2 occurred after 4 days of culture with FGF-2, specifically 15.7% and 11.5% of proteins became down-regulated upon 1 ng·mL⁻¹ and 5 ng·mL⁻¹ of FGF-2, respectively. On the other hand, compared to day 4, much less down-regulation took place at days 3 and 5. Only 11.7% and 12.8% of proteins were down-regulated upon treatment with 1 ng·mL⁻¹ at days 3 and 5, respectively. An even smaller proportion, 5.5% and 7.7% of proteins became down-regulated in cultures supplemented with

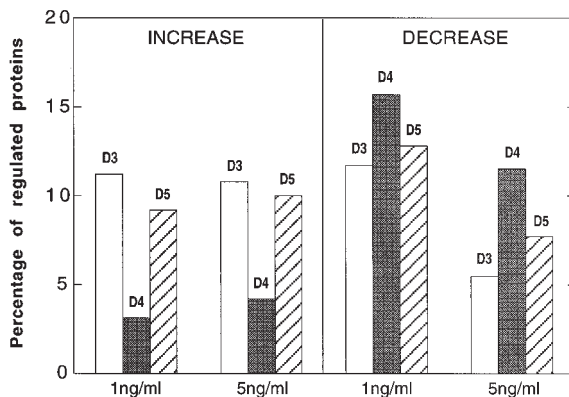


Figure 2. Number of at least two-fold up- and down-regulated protein spots as a percentage of spots resolved in the standard image for each of the three intervals and continual treatment of ICM-derived cells with FGF-2. All values are expressed as the mean percentage of two representative pairs of gels for 0 and 1 or 0 and 5 ng·mL⁻¹ of FGF-2. Note day 4 as a time window showing very characteristic changes in protein synthesis. The different fill patterns indicate D3, D4, and D5 of culture.

5 ng·mL⁻¹ of FGF-2. An example of FGF-2-induced changes in protein synthesis for each period of culture is shown in Figure 3.

Altogether, when ICM-derived cells were maintained in culture supplemented with FGF-2 for 4 days, that is, to the time point corresponding to day 8 of intrauterine development, they modulated the overall trend in protein synthesis in a way not observed in cells treated with FGF-2 for either 3 or 5 days.

3.2. Time-limited exposure to FGF-2 is sufficient to elicit a response of ICM-derived cells

The mechanism of growth factor-dependent embryonic inductive processes has several important limitations [10]. One of them is that the time for which tissue or cells can respond to signals may be restricted to a specific developmental period and, moreover, may be limited in its duration.

To test whether or not this scenario may apply to the specific changes in protein accumulation occurring in our in vitro system at day 4, we designed the following experiment. We cultured ICM-derived cells without FGF-2 for 80 hours and then we exposed the cells to 1 and/or 5 ng·mL⁻¹ FGF-2 for an additional 16 hours. Thus, these cells were labelled, lysed and subjected to 2D gel analysis at exactly the same time as cultures that were continuously treated with FGF-2 for 4 days beginning at the time of their isolation (Fig. 1). According to our standard criteria, it appears that short-term exposure to FGF-2 not only produces the significant effect, but its qualitative and quantitative parameters are essentially the same as in continuous exposure. Specifically, as shown in Figure 4, an average of 4.5% of proteins up-regulated and 12% of proteins down-regulated their accumulation upon short-term treatment with 1 and/or 5 ng·mL⁻¹ FGF-2.

4. DISCUSSION

Experimental design of our 2D gel analysis is based on a previously established model of in vitro mesoderm induction in the rabbit [5]. In that report we showed that culture of isolated ICMs in the presence of FGF-2 gives rise to migrating cells that are mesoderm-like in their phenotype. Their mesodermal phenotype was immunocytochemically demonstrated by the elevated expression of vimentin (days 7–10 of culture), *brachyury* (T) protein (day 10 of culture) and cytokeratins (day 10 of culture) and/or by down-regulation of desmoplakins (days 7–10 of culture) and embryoglycan epitope, stage-specific embryonic antigen 1 (SSEA 1/TEC 1; day 7–10 of culture). Correspondingly, mesoderm of entire rabbit embryo typically expresses *brachyury* (T) protein [12], cytokeratin and vimentin [27, 28]. Notably, our previous in vitro experiments pointed to the fact that increasing the concentration of exogenous FGF-2 results in changes of both proliferation activity and morphology of ICM-derived cells, and that these changes precede the expression of typical protein markers. In some cases, we observed the successive expression of vimentin in morphologically homogenous cell colonies (our unpublished data). We also observed that the expression of pluripotent cell-specific SSEA 1/TEC 1 was suppressed faster in the higher concentration of FGF-2, particularly in cells that migrate away from closely packed ectodermal or mesodermal colonies.

These previous observations led us to examine the overall quantitative pattern of protein synthesis using the same rabbit model. Two different concentrations of FGF-2 that we previously found to exert different effects on cell morphology and on the expression of protein markers were tested. Several lines of evidence make our hypothesis that overall protein synthesis may be dramatically changed during just three days of culture with FGF-2 biologically relevant. During the corresponding

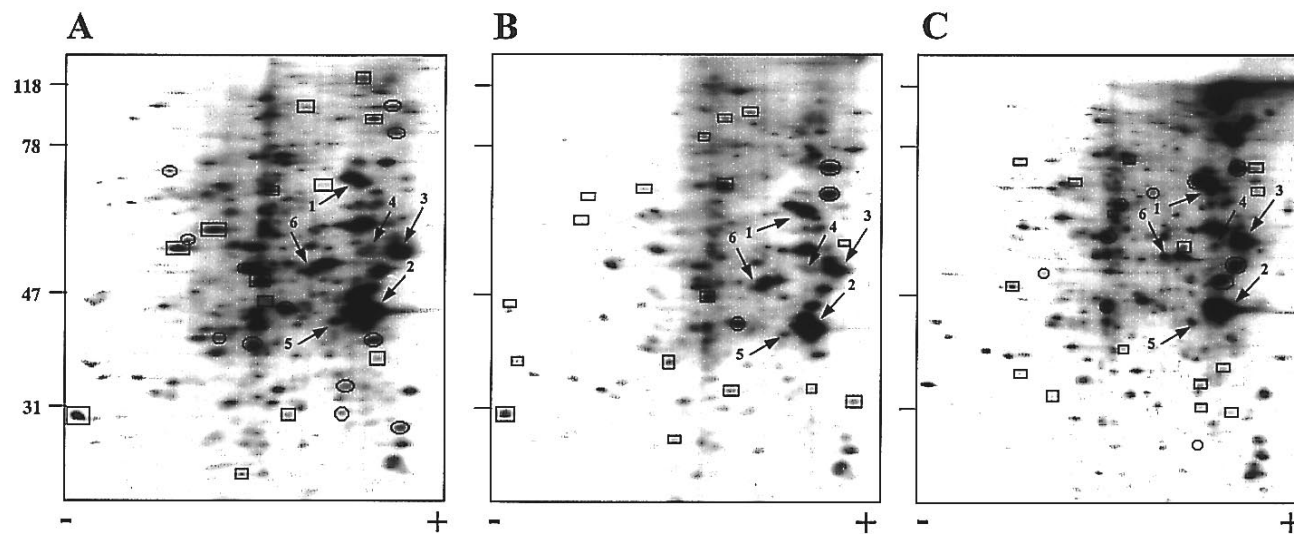
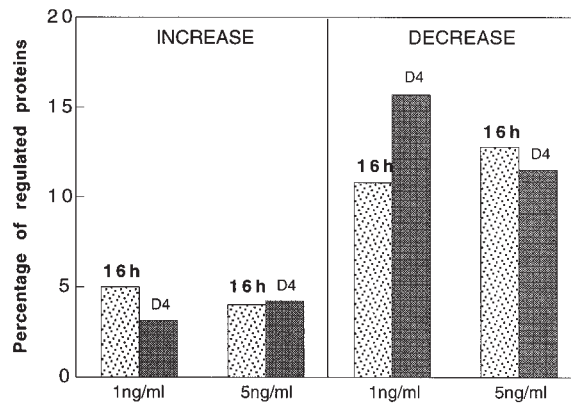


Figure 3. Autoradiographs of representative 2D gels of ICM-derived cells treated with 1 ng·mL⁻¹ of FGF-2 for 3 (A), 4 (B), and 5 (C) days. Proteins whose synthesis is altered upon FGF-2 are indicated by circles (increase) and squares (decrease), respectively. Numbered arrows point to a position expected for vimentin (1), cytokeratin 8 (2), and cytokeratin 18 (3). Molecular masses are given in kDa.

Figure 4. Number of at least two-fold up- and down-regulated protein spots as a percentage of spots resolved in the standard image for short-term exposure of ICM-derived cells to FGF-2 at day 4 of culture. All values are expressed as the mean percentage of two representative pairs of gels for 0 and 1 or 0 and 5 ng·mL⁻¹ of FGF-2. For comparison purposes, grey columns show the values obtained after continuous treatment with FGF-2 at day 4 of culture.



time period *in vivo*, mesoderm is induced and many primordial organs are formed. Clearly, this critical period of development is characterised by a rapid specification of cell lineages. This certainly involves transcriptional, translational and posttranslational regulation of the expression of many gene products. It is noteworthy that in the normally developing embryo, gradients other than FGF also play a role in driving various differentiation processes [4, 6]. Our culture system has the advantage of working with a fairly well-defined cell population – ICM, and we reduce external signals to probably one gradient – FGF-2. Therefore, this most likely leads to a dramatic simplification of both differentiating stimuli and cell response. It is also likely that some changes in protein synthesis do not necessarily result from treatment with FGF-2, but may reflect an adaptation of the ICMs to the culture conditions. Nevertheless, at least a tendency for growth factor-induced changes of protein synthesis in rabbit ICM-derived cells can be expected with some relevance to *in vivo* conditions.

Several previously reported *in vitro* experiments have suggested that growth factors regulate protein synthesis in preimplantation stage embryos. In isolated porcine embryonic discs more than 90% of the proteins increased their synthesis in the presence of IGF-1 [8]. In contrast, Shi et al. [20]

showed that in eight-cell and blastocyst stage mouse embryos the same growth factor, IGF-1, causes mostly inhibition of synthesis. These controversial data on corresponding developmental periods may suggest that regulation of protein synthesis by growth factors is species-specific.

Our analysis revealed a prominent time window in FGF-2-mediated regulation of protein synthesis/turnover. This distinguishable change in protein synthesis occurred at day 4 of culture. In the rabbit, this time is roughly equivalent to the major peak of organogenesis. Moreover, the percentage of proteins that were down-regulated was higher in the lower concentration of FGF-2. This suggests that two physiological doses of FGF-2 differ in their effects on regulation of protein synthesis. We put forward the hypothesis that concentrations of 1 and 5 ng·mL⁻¹ FGF-2 direct the differentiation of ectodermal cells into distinct cell lineages with their specific patterns of protein synthesis/turnover. Alternatively, our results may suggest that only a low dose of morphogen can induce true differentiation, while a higher dose rather pushes ectodermal cells towards the proliferation that can be exhibited by a less pronounced down-regulation of protein synthesis.

There are several other studies showing the changes in protein synthesis during

intrauterine development of the mouse that correspond to our results in the rabbit. In 7.5-day embryos, comparison of protein syntheses revealed the difference among embryonic and extraembryonic regions, and between isolated embryonic endoderm, mesoderm and ectoderm. In summary, these data revealed both up-regulation and down-regulation of protein synthesis with a predominantly increased rate of synthesis in the extraembryonic region and endoderm compared to embryonic region and ectoderm [15]. Changes in embryonic protein synthesis were also observed later in development, from day 7.5 to day 9. The protein synthesis was predominantly decreased when comparing day 7.5 with day 8 and day 8.5 with day 9. However, the synthesis in the time window between these stages, which coincides with the beginning of organogenesis (day 8 compared with day 8.5), was significantly increased [25]. Apparently, the mode of changes was rather quantitative, up- or down-regulation of existing proteins, although one protein, probably vimentin, was identified as being unique for anterior and posterior mesoderm and posterior ectoderm at day 7.5 [15] and several proteins were specifically expressed in cardiac components of the 9.5-day mouse [26] or in certain developmental stages [25].

Another result of our study is that none of the proteins that were shown by 2D gel electrophoresis to be significantly regulated upon FGF-2 clearly matched the mesodermal markers the expression of which was previously showed by immunocytochemistry. Thus, it is likely that the changes in protein synthesis that we revealed by short pulse radiolabelling and 2D analysis involve proteins that change their expression in advance of mesodermal markers mentioned above.

Taken together, our findings demonstrate that the candidate morphogen FGF-2 induces changes in rabbit embryonic cells that are well pronounced, show specific dynamics, and are coordinated by timely

restricted cell response. Future studies may employ amino acid sequencing to unravel the nature of some attractive candidate proteins.

ACKNOWLEDGEMENTS

This study was supported by a grant from the EU, BIO 2-CT 92-0358, and in part by grants 524/96/K162 from the Grant Agency of the Czech Republic and VZ432100001 from the Ministry of Education, Youth and Sports of the Czech Republic.

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