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The adhesion molecule NCAM promotes ovarian cancer progression via FGFR signaling

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision

07 December 2010

Thank you for the submission of your manuscript "The adhesion molecule NCAM promotes ovarian cancer progression via FGFR signaling". We have now heard back from the three referees whom we asked to evaluate your manuscript. You will see that they find the topic of your manuscript potentially interesting but they feel that the data need to be strengthened which should be addressed in a major revision.

In particular, reviewer #3 feels that the study would be significantly strengthened by additional evidence demonstrating that NCAM is needed for FGF-dependent metastasis. This reviewer also raises a number of technical issues.

Given the balance of the evaluations, we would be willing to consider a revised manuscript with the understanding that the reviewers' concerns must be convincingly addressed within the time constraints outlined below.

Should you decide to embark in such a revision, revised manuscripts should be submitted within three months of a request for revision. They will otherwise be treated as new submissions, unless discussed otherwise with the editor.

I look forward to seeing a revised form of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1 - Comments on Novelty/Model system:

This is a solid study, however the concepts presented are neither completely novel nor sufficiently translationally extended as to be of major impact currently, and extend knowledge rather than present completely novel concepts. The models used are adequate, but really use only one human cell line, and could benefit from other examples in-vivo.

Referee #1 - Other Remarks:

This is a worthy study that applies concepts broadly already published elsewhere to ovarian cancer. The main novel finding is the regulation of peritoneal dissemination by NCAM via FGFRs, which is broadly predictable given the previous literature (including previous analysis of NCAM in human ovarian cancer and advances the field a little.

Referee #2:

EMM-2010-00539-T

The adhesion molecule NCAM promotes ovarian cancer progression via FGFR signaling

Summary:

This is a well-written, carefully executed study for which there is very little criticism. Expression of NCAM was evaluated by immunohistochemical staining in a large collection of human epithelial ovarian cancers and in normal ovaries and a limited number of benign cystadenomas. This analysis showed that NCAM is not detected in normal ovarian epithelium or benign lesions, but is detected in ~24% of primary EOC and ~35% of EOC lesions spread beyond the ovary, suggesting a role in tumor progression. The authors conducted additional in vitro experiments with EOC cell lines, using multiple strategies for modulation of NCAM activity, including RNA interference in cells expressing high levels of NCAM and stable expression of full length NCAM and a construct lacking the FN2 domain in cells expressing little or no detectable NCAM. The study also utilizes multiple approaches for inhibition (pharmacologic agent, dominant negative FGFR, function blocking anti-NCAM antibodies) and activation (activating peptide) to demonstrate the functional interaction of NCAM with FGFR in mediating EOC cell motility and invasion. Finally, overexpression of NCAM resulted in significantly increased peritoneal tumor dissemination in a pseudo-orthotopic xenograft model of EOC. This increase in tumor dissemination could be inhibited by a function blocking anti-NCAM monoclonal antibody. Collectively, the results identify NCAM-mediated FGFR signaling as a potential therapeutic target/pathway that may be exploited to inhibit tumor cell motility and invasiveness.

Specific comments:

1. The first two sentences of the Discussion are seemingly at odds. If the results are in line with a previous publication in 2006 showing a lack of NCAM expression in normal epithelium and high level of enrichment in advanced EOC, the meaning of the first sentence "This study reports for the first time" Is unclear. Please clarify.
2. There are two minor grammatical errors in the Introduction on page 5.
 - a. Paragraph 1, second sentence should read: " The deregulation of FGFR signaling in cancer can result from different..."
 - b. Paragraph 2, first sentence should read: "Base on the ability of NCAM to modulate FGFR function..."

Referee #3 - Comments on Novelty/Model system:

The in vitro system is adequate and the chosen controls are appropriate. While the ip xenograft model has been previously used to assess tumorigenicity, this model has limitations appreciating the role of proteins implicated in migration and invasion, such as NCAM and FGFR. An ovarian orthotopic model that can appreciate the movement of tumor cells from the primary tumor site to secondary sites would replicate more accurately the steps of metastasis.

Referee #3 - Other Remarks:

In this manuscript, Dr. Zecchini and colleagues describe the role of NCAM and its interaction with FGFR in regulation of cell migration and invasion, and the peritoneal dissemination of ovarian tumors. The manuscript is well written and by and large experiments are well designed and controlled. The findings have clinical relevance. While the study of NCAM's expression and function in ovarian cancer is new, its interaction with FGFR and implications for cellular migration in other systems have been known.

Major concerns:

- 1) It is not clear from the data presented whether the function of NCAM adds a new dimension to the known role of FGFR in cancer. In other words, while the authors present convincing data that FGFR is necessary for NCAM's function, it is not clear whether NCAM is needed for FGF-dependent signaling to affect cellular migration and metastasis.
- 2) The ip xenograft used may not be the optimal model to study how a protein involved in cellular migration affects cancer metastasis. An orthotopic model is better suited to address this question.
- 3) Given that the interaction between FGFR and NCAM is essential for NCAM's functions, is there a correlation between FGFRs and NCAM expression in ovarian tumors (Figure 1)?

Minor concerns:

- 1) As AG1478 is used as a negative control, is it known whether MOVCAR cells express the EGFR (Figure 2)?
- 2) Data in Figures 2A and 2B suggest that FGFR inhibition affects migration and invasion independent of NCAM.
- 3) Why does inhibition of EGFR increase cellular migration of cells in which NCAM was knocked down compared to control (figure 2B)?
- 4) Re the ip xenograft model: the number of bowel metastases in the control arm (SKOV3) appears lower than what is typically expected with these cells (~2 per field, reported here).
- 5) Does NCAM overexpression affect tumor volume in xenograft models?
- 6) Is there previous evidence or support for quantifying tumor metastasis on liver and diaphragm as the percentage of GFP+ cells of a mixed cell population derived from digested tissue? Counting tumor implants, as done for bowel, may be a more direct estimation of metastasis.
- 7) Demonstrating direct interaction between FGFR and NCAM in ovarian cancer cells would support the model proposed.

POINT-BY-POINT REPLIES TO REVIEWERS

Referees' comments are in italic style. Our replies are in regular style.
The modifications made in the revised version of the manuscript are written in red.

Referee #1 (Comments on Novelty/Model system):

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This is a worthy study that applies concepts broadly already published elsewhere to ovarian cancer. The main novel finding is the regulation of peritoneal dissemination by NCAM via FGFRs, which is broadly predictable given the previous literature (including previous analysis of NCAM in human ovarian cancer) and advances the field a little.

We share with this Referee the view that the functional interplay between NCAM and FGFR does not represent a completely novel concept. Indeed, our group itself has contributed to elucidate the molecular mechanisms and the signaling pathways related to the NCAM/FGFR complex. Nevertheless, this manuscript provides the first demonstration that the interaction of NCAM with FGFR has important biological implications and, in particular, is causally involved in ovarian cancer progression. Furthermore, we believe that the inhibition of cancer dissemination upon targeting the NCAM/FGFR interplay has novel and relevant translational implications.

We respectfully disagree with the Referee's opinion that our findings were "broadly predictable" based on the previous literature. For example, in the Rip1Tag2 mouse model of pancreatic beta cell tumorigenesis the interaction of NCAM with FGFR prevents metastatic dissemination due to its ability to promote tumor-cell matrix adhesion and, hence, to prevent cell detachment from the primary tumor. Notably, in tumor cells derived from Rip1Tag2 mice NCAM failed to induce migration or invasion (Cavallaro et al., Nat Cell Biol, 2001). Together with the observation that NCAM expression in cancer can be dramatically up or downregulated depending on the tumor type (Zecchini and Cavallaro, Adv Exp Med Biol, 2010), this suggests that the effect of NCAM/FGFR on cancer cell behavior is context and tumor type-dependent. There is no information on the possible role of the NCAM/FGFR interplay in ovarian cancer biology, and what has been published on this interplay in neural cells (Doherty's studies) or in mouse fibroblasts and HeLa cells (our work) is not automatically applicable to ovarian carcinoma. Along the same line, the results of the previous analysis of NCAM expression in this tumor type do not imply at all that a functional interaction of NCAM with FGFR underlies cancer progression.

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EMM-2010-00539-T

The adhesion molecule NCAM promotes ovarian cancer progression via FGFR signaling

Summary:

This is a well-written, carefully executed study for which there is very little criticism. Expression of NCAM was evaluated by immunohistochemical staining in a large collection of human epithelial ovarian cancers and in normal ovaries and a limited number of benign cystadenomas. This analysis showed that NCAM is not detected in normal ovarian epithelium or benign lesions, but is detected in ~24% of primary EOC and ~35% of EOC lesions spread beyond the ovary, suggesting a role in tumor progression. The authors conducted additional in vitro experiments with EOC cell lines, using multiple strategies for modulation of NCAM activity, including RNA interference in cells expressing high levels of NCAM and stable expression of full length NCAM and a construct lacking the FN2 domain in cells expressing little or no detectable NCAM. The study also utilizes multiple approaches for inhibition (pharmacologic agent, dominant negative FGFR, function blocking anti-NCAM antibodies) and activation (activating peptide) to demonstrate the functional interaction of NCAM with FGFR in mediating EOC cell motility and invasion. Finally, overexpression of NCAM resulted in significantly increased peritoneal tumor dissemination in a pseudo-orthotopic xenograft model of EOC. This increase in tumor dissemination could be inhibited by a function blocking anti-NCAM monoclonal antibody. Collectively, the results identify NCAM-mediated FGFR signaling as a potential therapeutic target/pathway that may be exploited to inhibit tumor cell motility and invasiveness.

Specific comments:

1. The first two sentences of the Discussion are seemingly at odds. If the results are in line with a previous publication in 2006 showing a lack of NCAM expression in normal epithelium and high level of enrichment in advanced EOC, the meaning of the first sentence "This study reports for the first time" Is unclear. Please clarify.

The words "for the first time" alluded specifically to the absence of NCAM in normal ovarian surface epithelium, since this tissue was not analysed in the previous publication. Nevertheless, to avoid any confusion, we have removed those words from the first sentence of the Discussion.

2. There are two minor grammatical errors in the Introduction on page 5.
a. Paragraph 1, second sentence should read: " The deregulation of FGFR signaling in cancer can result from different..."
b. Paragraph 2, first sentence should read: "Based on the ability of NCAM to modulate FGFR function..."

Both errors have been corrected.

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Major concerns:

1) *It is not clear from the data presented whether the function of NCAM adds a new dimension to the known role of FGFR in cancer. In other words, while the authors present convincing data that FGFR is necessary for NCAM's function, it is not clear whether NCAM is needed for FGF-dependent signaling to affect cellular migration and metastasis.*

We agree with the Referee that this is a relevant issue. Our previous data have indicated a profound dichotomy between the FGFR responses elicited by NCAM and FGF. Indeed, not only the two ligands induce the activation of different downstream effectors, but also the signal duration and the intracellular trafficking of FGFR are dramatically different. We have also demonstrated in different cell types that FGF promotes cell proliferation but not migration, while NCAM induces FGFR-dependent migration but not proliferation (Francavilla et al., J Cell Biol, 2009). Finally, we have reported that NCAM inhibits FGF activity, rather than cooperating with it. This is due to NCAM's ability to compete with FGF for the binding to its receptor (Francavilla et al., J Cell Sci, 2007). These observations, as outlined in the Discussion (from p. 19, line 20), lend support to the notion that FGF does not cooperate with NCAM in the stimulation of FGFR signaling.

Furthermore, our results showed that FGF itself has a negligible influence on the migration of wild-type SKOV3 cells (compare the green bars in the figure below) (Figure not included in this Review Process file.) The ectopic expression of NCAM, while resulting *per se* in a strong induction of cell migration, does not modify the lack of migratory response to FGF (compare the red bars in figure below), thus confirming that FGF is not involved in NCAM/FGFR-dependent EOC cell migration.

FGF-2 also failed to enhance migration in MOVCAR cells (not shown) as well as in ID8, another mouse EOC cell line that expresses high levels of NCAM (see figure below). We tested whether ablating NCAM had any effect on cell migration in FGF-2-stimulated EOC cells. As shown in the figure below, NCAM gene silencing in ID8 cells resulted in decreased migration, in agreement with our observations in MOVCAR cells (Fig. 2 in the manuscript). FGF-2 treatment showed no influence on the migratory ability of NCAM-deficient ID8 cells (figure below, right panel) (Figure not included in this Review Process file.) These data confirm that FGF is unable to affect migration in various EOC cell lines, and NCAM does not modify this lack of pro-migratory activity, thus confirming and complementing the above-mentioned data on SKOV3 cells.

These findings and the fact that they rule out a cooperation between NCAM and FGF in EOC cell migrations are now discussed in the Results (p. 9, lines 14-17; p. 11, line 24, to p. 12, line 3).

2) *The ip xenograft used may not be the optimal model to study how a protein involved in cellular migration affects cancer metastasis. An orthotopic model is better suited to address this question.*

We thank the Referee for raising this point.

Given the intrinsic technical difficulty of an orthotopic mouse model of ovarian carcinoma (i.e., injection of EOC cells in a very small volume under the mouse ovarian bursa), the ip xenograft is widely used as a faithful model of EOC metastasis (see for example Mitra et al., Oncogene, 30:1566–1576, 2011; Huang et al., PNAS, 106:3426-3430, 2009; Yu et al., J Natl Cancer Inst, 20:1630-1642, 2008). Other references on the specific use of SKOV3 cells in this model are cited in the Results in support of our approach (p. 15, lines 12-13).

Nevertheless, to address the Referee's issue, we have devoted a major effort to establish and optimize the orthotopic mouse model of EOC with SKOV3 cells transfected with NCAM, either full-length or DFN2, or with an empty vector. In particular, tumor development was analyzed at two weeks, to assess local invasion, and at 9 weeks, to assess metastatic dissemination to peritoneal organs. In agreement with the results obtained *in vitro* and with the i.p. xenograft model, NCAM induced local invasion of SKOV3 cells within the ovarian parenchyma. In addition, NCAM-positive tumors invaded the surrounding, extra-ovarian soft tissue, further supporting the role of NCAM in EOC invasion and dissemination. None of these events was observed with DFN2-transfected cells,

confirming their dependence on the NCAM/FGFR interaction. Finally, intravascular tumor lesions were observed in mice injected with NCAM-expressing SKOV3 but not with mock or DFN2 cells, thus suggesting that NCAM also enhances EOC dissemination through the circulation.

While these findings support the role of NCAM in EOC metastatic spread to peritoneum, the latter process could not be assessed properly in the SKOV3 orthotopic model, due to its intrinsic high degree of variability. Indeed, in our experience, intra-bursal injection of SKOV3 cells (no matter whether they express NCAM or not) results in rapid growth within the ovary and can give rise to large cystic tumors (up to 2 grams of weight and 20x15mm in size). In over 20-40% of the experimental animals these cysts have already ruptured by the 9th week after inoculation and have released large amounts of tumor cells and clusters throughout the abdominal cavity. The inconsistent rate of tumor growth within the ovary (a problem inherent to this type of experiment) and the event of cyst rupture, most likely account for the high variability that we observed in the number of peritoneal metastases assessed at this time point.

To address this Referee's comment, we have assessed metastatic dissemination to the contralateral ovary, the liver and the diaphragm, 9 weeks after orthotopic SKOV3 cell inoculation. Upon normalization against the volume of the primary tumor, the data shows that NCAM, but not its DFN2 mutant, promotes metastatic dissemination to the liver and to the diaphragm (see figure below). However, the results did not reach statistical significance due to the above-mentioned variability.

We have therefore added to the manuscript only the data on local invasion and dissemination in the orthotopic model, i.e. those referring to the 2-week time point (Figs. 5, S7 and S8; the results are discussed on p. 14-15), while for peritoneal metastases we have kept the results of the i.p. xenotransplantation experiments that were submitted originally.

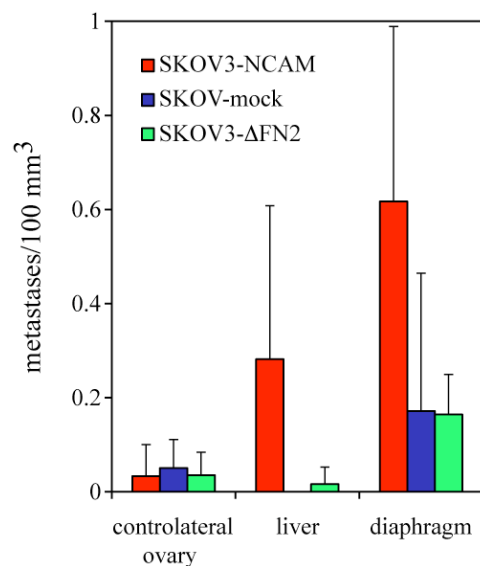


Figure legend. SKOV3 were injected under the ovarian bursa. Primary tumors and metastasis to contralateral ovary, liver and diaphragm were assessed 9 weeks after the injection. The number of metastases was normalized by the volume of the primary tumor. Five mice per group were used. Data represent means \pm SD.

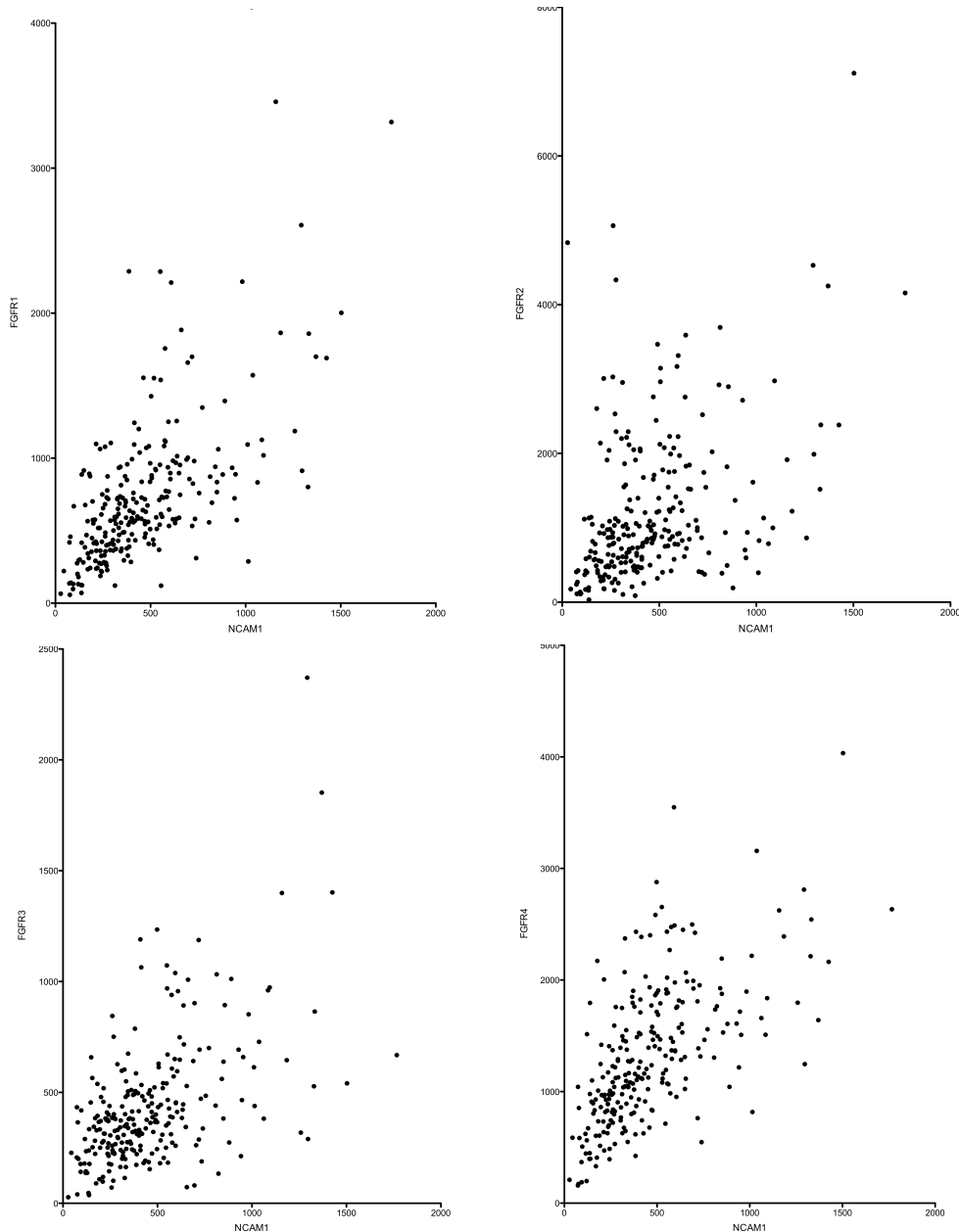
3) Given that the interaction between FGFR and NCAM is essential for NCAM's functions, is there a correlation between FGFRs and NCAM expression in ovarian tumors (Figure 1)?

We have subjected a cohort of 82 EOC samples to immunohistochemical staining for FGFR1 and NCAM. Over 97% percent of the samples (75 out of 77) scored positive for FGFR1 and 30% (23 out of 77) were positive for NCAM. This implies that every tumor where NCAM is present expresses also FGFR1 (and likely other FGFRs; for example FGFR2 and FGFR4 have been found in at least 80% of EOC; Steele et al., *Oncogene*, 2001; Valve et al., *Int J Cancer*, 2000), thus supporting the hypothesis that NCAM stimulates FGFR activity in EOC tissue. These data are shown in Table S1 and Fig. S1, and discussed in the Results (from p. 7, line 23 to p. 8, line 2).

Furthermore, we analysed the co-expression of the NCAM1 gene with members of the FGFR family in published microarray datasets of a cohort of 255 ovarian cancer patients (Crijns et al. Survival-related profile, pathways, and transcription factors in ovarian cancer. *PLoS Med*, 6, e24, 2009).

Microarray data analysis indicated a significant degree of correlation (Pearson's R^2 coefficient range 0.4 - 0.65) between the normalized expression values of NCAM1 and each FGFR gene (FGFR1, FGFR1, FGFR3 and FGFR4). Representative graphs indicating the correlations of individual FGFRs with NCAM in EOC microarray datasets are shown in the figure below ($p < 0.0001$).

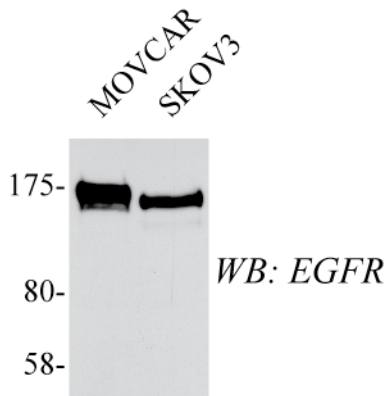
Analysis of the frequency distribution of the expression values showed that high NCAM expression is always associated with FGFR1, FGFR2 and FGFR3 expression (for all genes, the top 25% percentile was arbitrarily considered "highly expressing" and the lower 25% percentile "negative"). These data are mentioned in the Results as "data not shown" (p. 8, lines 2-7).



Minor concerns:

1) As AG1478 is used as a negative control, is it known whether MOVCAR cells express the EGFR (Figure 2)

Our immunoblotting analysis confirmed the expression of EGFR in both MOVCAR and SKOV3 cells (see figure below). The expression of EGFR is now mentioned in the Results as "data not shown" (p. 9, line 19).



2) Data in Figures 2A and 2B suggest that FGFR inhibition affects migration and invasion independent of NCAM.

We respectfully disagree with the Referee's interpretation of the data. In Fig. 2A and 2B, PD173074 inhibits cell migration and invasion only on cells that express NCAM, while no effect is observed in cells where NCAM expression has been silenced. Therefore, FGFR inhibition is effective only in the presence of NCAM, in line with the notion that NCAM stimulates EOC cell migration and invasion via FGFR.

3) Why does inhibition of EGFR increase cellular migration of cells in which NCAM was knocked down compared to control (figure 2B)?

We should point out that the difference in MOVCAR-shNCAM cell invasion between untreated and AG1478-treated cells is not statistically significant ($p=0.06$; this information has now been included in the legend to Fig. 2B, p. 38, lines 16-18). The apparent increase in cell invasion upon AG1478 treatment, therefore, appears to depend on inter-experimental variability.

Nevertheless, MOVCAR-shNCAM cells show indeed a trend towards increased invasion upon EGFR inhibition. Since the effect of EGFR inhibition on MOVCAR cell invasion is out of the scope of our study, we have not investigated that phenomenon and cannot offer a clear answer to the Referee's question. EGFR has been shown to reduce neurite outgrowth and NCAM promotes EGFR degradation in neural cells (Povlsen et al., J Neurochem, 2008). Along that line, one can speculate that EGFR signaling has a negative impact on MOVCAR cell invasion and that NCAM reduces the levels of EGFR in those cells. If this were the case, the combination of NCAM knockdown (leading to increased EGFR and, hence, to inhibition of invasion) with AG1478 treatment (abolishing the negative effect of EGFR) would result in increased cell invasion, thus accounting for the trend shown in Fig. 2B.

It should be emphasized that, even if AG1478 would really influence MOVCAR cell migration, that phenomenon could not be generalized, since for example AG1478 has no effect on the migration of SKOV3 cells (Fig. 3B).

4) Re the *ip xenograft model*: the number of bowel metastases in the control arm (SKOV3) appears lower than what is typically expected with these cells (~2 per field, reported here).

We regretfully admit that we do not understand what this comment refers to, although we assume that the Referee is alluding to Fig. 5B.

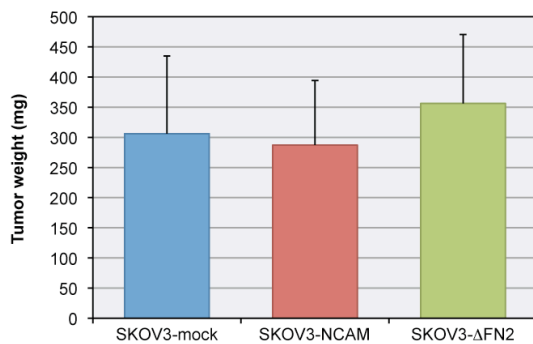
The number of bowel metastases per field is strictly dependent on the specific experimental conditions used (number of injected cells, time length of *in vivo* assay, imaging conditions such as microscope objective, zoom, etc.). Therefore, it is quite difficult to establish what is a standard number of metastases that one can "typically expect". Nevertheless, we have now added to

Materials and Methods more technical details on how the number of metastases was determined (from p. 28, line 23, to p. 29, line 2).

In case “the control arm” mentioned by the Referee indicates SKOV3-DFN2 tumors, it is indeed true that with these cells we observed a lower number of bowel metastases/field as compared to SKOV3-mock cells. At this stage, we cannot provide an explanation for that decrease, neither can we establish whether it has any biological significance. The reduction in SKOV3-DFN2 bowel metastasis is now mentioned in the Results (p. 16, lines 1-4).

5) *Does NCAM overexpression affect tumor volume in xenograft models?*

We thank the Referee for raising this point that we had erroneously omitted in the first version of the manuscript. NCAM overexpression in SKOV3 cells does not affect tumor burden in the xenograft mouse model. We have determined the weight of the tumor mass in the mouse omentum, which is the primary site of colonization upon i.p. injection of SKOV3 cells (see for example Sawada et al., Cancer Res, 2008). As shown in the figure below, no significant changes were observed when SKOV3-mock tumors were compared with SKOV3-NCAM or SKOV3-DFN2 tumors (n=5). This information has been added to the Results as “data not shown” (p. 15, lines 15-18).



6) *Is there previous evidence or support for quantifying tumor metastasis on liver and diaphragm as the percentage of GFP+ cells of a mixed cell population derived from digested tissue? Counting tumor implants, as done for bowel, may be a more direct estimation of metastasis.*

We have undertaken the FACS-based approach based on a number of reports where the percentage of GFP+ tumor cells within a mixed cell population was used to quantify metastasis in target organs including liver, lung, brain, lymph nodes (see for example Lagadec et al., Oncogene, 2009; Shannon et al., Clin Exp Met, 2004; Schmidt et al., Clin Exp Met, 1999; Hirakawa et al., J Exp Med, 2005).

Nevertheless, to address this Referee’s point, we have repeated the *in vivo* assay and counted the GFP+ tumor implants on the liver and the diaphragm. This approach confirmed the results of the previous experiments, with NCAM-expressing cells forming more metastases in both organs (although the increase in liver metastases did not reach statistical significance). This new set of data has been included in the Results (Fig. 6C) and the relevant text of the figure legend and of Materials and Methods has been corrected accordingly (p. 40, lines 17-19; p. 29, lines 2-3).

07) *Demonstrating direct interaction between FGFR and NCAM in ovarian cancer cells would support the model proposed.*

We have performed co-immunoprecipitation assays in SKOV3 cells, and found that wild-type NCAM, but not DFN2, associates with FGFR1. These results are now shown in Fig. S5A and discussed in the Results (p. 11, lines 13-14).

Thank you for the submission of your revised manuscript "The adhesion molecule NCAM promotes ovarian cancer progression via FGFR signaling" to EMBO Molecular Medicine. We have now received the enclosed report from the referee that was asked to re-assess it. As you will see the reviewer is now globally supportive and I am pleased to inform you that we will be able to accept your manuscript pending the following final amendments:

As highlighted by the Reviewer, we would encourage you to include the correlation analysis shown in the Response to Reviewers in the Supplementary Information and state the correlation coefficient in the manuscript.

On a more editorial note, please be aware that the manuscript must also include an ethics statement regarding the compliance with relevant guidelines and regulations (in this case informed consent of patients, please see excerpt from the Guide to Authors below).

We also note that the Response to Reviewers contains additional figures, which would be published in the Review Process File (for more information on our transparent editorial process, please see below). Please indicate whether you agree with their publication or would like to exclude them.

Please submit your revised manuscript within two weeks. I look forward to seeing a revised form of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #3 (Comments on Novelty/Model system):

the study is not that novel, as the NCAM/FGFR interaction is already known. Application to ovarian cancer is new.

Referee #3 (Other Remarks):

The authors have addressed satisfactorily most of the questions raised. Addition of the orthotopic model strengthens the manuscript, although effect on peritoneal metastasis is only marginally explored with this model (no quantification provided). Since NCAM is expressed in only 25-30% of tumors, generalizing its functions to the entire ovarian cancer population remains questionable.

Minor comments:

1) Text can be condensed. Example: eliminate sentence page 6, lane 13 "FGFR attracted the attention of many investigators" as it does not convey information. There are other parts of the manuscript that can be condensed.

2) If a significant correlation between NCAM and FGFR is mentioned (page 10, lane 4), then the coefficient of correlation should be noted and data enclosed as supplementary information.

2nd Revision - authors' response

27 May 2011

Thank you very much for your decision letter on our manuscript.

I have prepared a new version of the Supplementary Information which includes the correlation analysis. Also, I have added the information on

the informed consent of the patients in the Materials and Methods section. As agreed yesterday by telephone with the editorial assistant yesterday, I have sent the new files by email. I hope that the manuscript will now be considered suitable for publication.

With regard to your question about publishing the figures included in our Response to Reviewers, I would like to exclude the first two figures.

Thank you very much and I'm looking forward to hearing from you soon.