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TITLE: Role of Osteopontin in Hepatocellular Carcinoma

PRINCIPAL INVESTIGATOR: Natalia Nieto

CONTRACTING ORGANIZATION: The Board of Trustees of The University of Illinois
The University of Illinois at Chicago

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PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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6. AUTHOR(S)
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The Board of Trustees of The University of Illinois
The University of Illinois at Chicago

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U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
This project aims to dissect the molecular mechanisms whereby Osteopontin (OPN) drives hepatocellular carcinogenesis and progression, to fill the gap in our knowledge on the pathogenesis of hepatocellular carcinoma (HCC). Our main hypothesis was that secreted OPN signals via autocrine and paracrine activation of the CD44 receptor. We also hypothesized that OPN induces the emergence, maintenance and progression of cancer stem cells (CSCs) by activating CD44. Our results show that secreted OPN has limited impact on CSCs as well as on carcinogenesis in general and that CD44 is not involved in the effects of OPN. However, we found that ablation of *Opn* in hepatocyte represses the early response to DNA damage and drives the emergence of CSCs and the subsequent HCC. Overexpression of OPN in hepatocyte has less impact on the response to DNA damage and does not increase the emergence of CSCs but it drives their phenotype toward carcinogenesis.

15. SUBJECT TERMS Hepatocellular carcinoma, Osteopontin, CD44, p53					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U	UU	17	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

Our **overall objective** was to dissect the molecular mechanisms whereby Osteopontin (OPN) drives hepatocellular carcinogenesis and progression, to fill the gap in our knowledge on the pathogenesis of hepatocellular carcinoma (HCC), a disease affecting the general population, which has a particularly profound impact on the health and well-being of Military Service members and US Veterans.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

Hepatocellular carcinoma, Osteopontin, cancer stem cells, diethylnitrosamine, CD44, p53.

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Our **specific aim 1** was to dissect if OPN binding to CD44 in hepatocytes inhibits DNA repair, apoptosis and the cell cycle by blocking p53, using the diethylnitrosamine (DEN) model at early time points (24 and 48 h). Specific aim 1 was accomplished during the first 6 months of the project.

The **specific aim 2** was to establish if hepatocyte-derived OPN stimulates the emergence of cancer stem cells (CSCs) and increases their maintenance and proliferation. Specific aim 2 was accomplished during months 5 to 20 of the project.

What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

All the experiments scheduled in our application were performed.

In **Aim 1**, we measured OPN secretion after DEN injection, to support our hypothesis that OPN is secreted and activates CD44 receptor following DNA damage (induced by DEN). However, we did not find a significant difference in OPN levels after DEN.

Next, we focused on p53 signaling after DEN injection in 4 groups of mice: wild-type (WT), *Opn* knockout in hepatocytes ($Opn^{\Delta Hep}$), *Opn* transgenic in hepatocytes (Opn^{Hep} Tg) and $Cd44^{-/-}$. To this end, we measured mRNA expression of p53 targets by qPCR and assessed p53 protein expression and phosphorylation by Western blot. We did not find any effect of DEN injection, *Cd44* ablation or of *Opn* overexpression or ablation on p53 protein expression. Nonetheless some p53 target genes were affected, particularly in $Opn^{\Delta Hep}$ mice at 48 h. This encouraged us to perform RNAseq in $Opn^{\Delta Hep}$ and Opn^{Hep} Tg mice, injected with DEN or PBS at 48 h. We found a very strong cellular response after DEN injection in WT mice at 48 h, with 1,347 differentially expressed genes (951 upregulated and 396 downregulated). This response was further characterized using the Ingenuity Pathway Analysis (IPA) platform. There was significant metabolic reprograming (activation of oxidative phosphorylation, TCA cycle and lipid synthesis, reduced cholesterol and unfolded protein response), activation of mTOR, cJun and p53 signaling and decreased cell cycle. To our knowledge, this is the first time that metabolic reprograming is shown in response to DNA damage. We next focused on the effect of *Opn* ablation or overexpression and found that in $Opn^{\Delta Hep}$ mice, the response to DEN is considerably reduced (1,281/1,347 of the differentially expressed genes were not affected by DEN). In Opn^{Hep} Tg mice, there was partial protection from the response to DEN (822/1,347 of the DE genes were not affected by DEN) **(Figure 1)**.

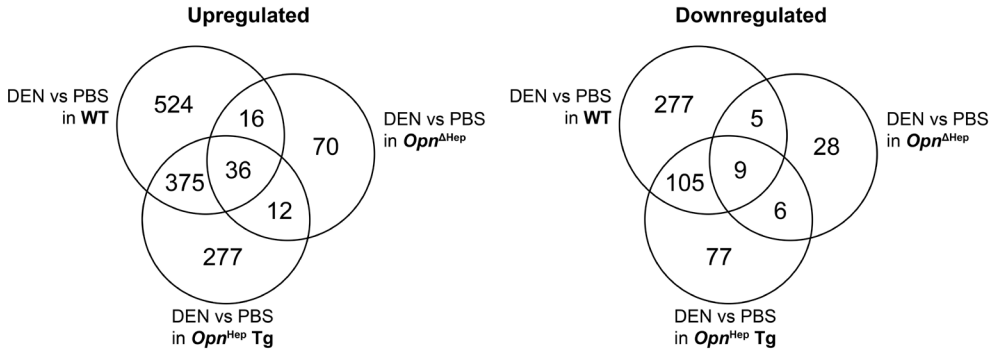

Figure 1. Venn diagram showing the number of differentially expressed genes in 3 groups of mice after DEN injection, based on RNAseq at 48 h.

The role of this cellular response to DEN is unknown but this could potentially protect the cell against carcinogenesis either by inducing DNA repair or by affecting DNA methylation.

To test this possibility, we analyzed publicly available human data to determine whether OPN expression is associated with a change in the number of mutations or with a change in DNA methylation in HCC patients. We did not find any difference in DNA mutations but found that human HCC with elevated OPN had more frequent DNA hypomethylation. Hence we hypothesized that the cellular response to DEN could affect DNA methylation to protect cells against carcinogenesis and that reduction of this response following *Opn* ablation and/or overexpression could facilitate the emergence of HCC. To test this hypothesis, we measured global DNA methylation using the LINE1 assay at different time points following PBS or DEN injection in different groups of mice **(Figure 2)**. The results showed that DEN does not affect DNA methylation in normal liver (HCC showed

higher DNA methylation only in WT mice). We found a small decrease in global DNA methylation in *Opn*$^{\Delta Hep}$ mice, only in HCC but it was not significant.

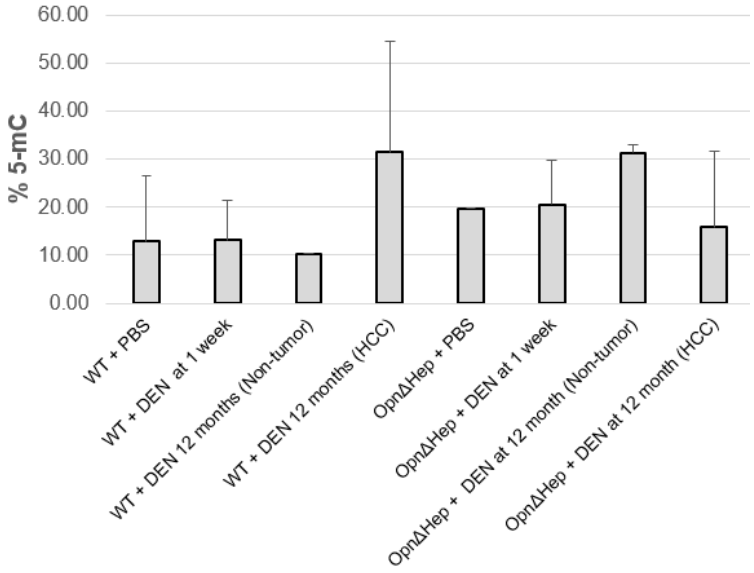

Figure 2. Global level of DNA methylation in WT and *Opn*$^{\Delta Hep}$ mice following DEN injection.

In **summary**, we found that *Opn* ablation, and to a lesser extent *Opn* overexpression, represses the early response to DEN, which could affect carcinogenesis. However, we were unable to find a mechanism linking the early response to DEN to carcinogenesis. This effect was not mediated by *Opn* secretion, as *Opn* was not increased following DEN injection, or by CD44, as *Cd44* ablation did not affect the response to DEN (based on qPCR analysis). The effect could be mediated by p53, because p53 signaling was activated at the mRNA level following DEN injection, but this is unlikely as the total and phosphorylated p53 did not change by DEN injection. Last, the effect was not mediated by DNA methylation because we did not find a change in DNA methylation after DEN injection.

In **Aim 2**, we first analyzed the number of CSCs in 3 groups of mice: WT, *Opn*$^{\Delta Hep}$ and *Opn*^{Hep} Tg. CSCs can be detected in the aggregate fraction of primary hepatocytes by co-expression of the progenitor markers CD44 and AFP. Five months after DEN injection, there was a significant increase in the number of CSCs in *Opn*$^{\Delta Hep}$ mice but not in *Opn*^{Hep} Tg mice **(Figure 3)**. This indicates that *Opn* ablation but not overexpression drives the early emergence of CSCs.

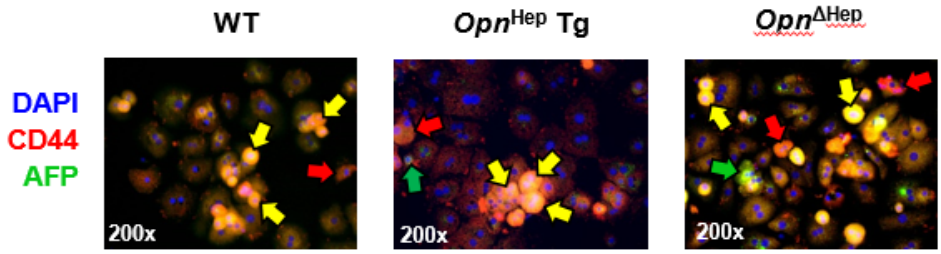

Figure 3. Immunofluorescence of CD44 (red) and AFP (green) (markers of CSCs) in the aggregate fraction of primary hepatocytes in 3 groups of mice 5 months after DEN injection. Red arrow: CD44^{+}AFP^{-} cells; green arrow: CD44^{-}AFP^{+} cells; Yellow arrow: CD44^{+}AFP^{+} cells.

Next, we hypothesized that secreted OPN in *Opn*^{Hep} Tg mice could affect the phenotype of CSCs and promote their progression to cancer by activating CD44. To test this, we first confirmed that *Opn*^{Hep} Tg mice have higher secretion of OPN by measuring OPN in the serum. Next, we collected CSCs from WT mice 5 months after DEN injection and treated them with recombinant OPN (rOPN) for 24 h. We then performed qPCR to evaluate the expression of markers of proliferation, HCC and stemness. However, we did not find any change. To go further, we performed RNAseq to identify the signaling pathways affected by rOPN but we found no differentially expressed genes. This indicates that secreted OPN has little impact on the CSCs phenotype.

To confirm this, we transplanted CSCs into mice with different levels of OPN to study the impact of extracellular OPN on CSCs progression. We isolated CSCs from 5-months-old DEN-injected WT mice and transplanted them via intrasplenic injection into WT or *Opn*$^{-/-}$ recipient mice. One week later, recipient mice were treated with CCl_4 for 3 weeks to induce fibrosis (necessary for this model to work) but also to stimulate OPN secretion in WT but not in *Opn*$^{-/-}$ mice. MRI at 5 and 8 months showed small (<2 mm) and large (>10 mm) tumors, respectively, in both groups. After 8 months, most mice developed at least one tumor but there was no difference between groups **(Figure 4)**. Importantly, the tumor tissue from *Opn*$^{-/-}$ mice expressed OPN, confirming that HCC arose from transplanted CSCs originated in WT mice.

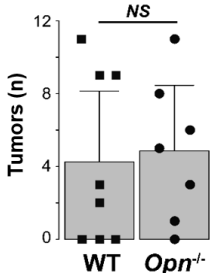

Figure 4. Number of macroscopic tumors per mouse liver from WT (n=8) and *Opn*$^{-/-}$ (n=7) mice 8 months after CSCs transplantation (mean ± SEM).

All these results suggest that secreted OPN does not play a major role in carcinogenesis, but indicate that intracellular OPN could. To study the role of elevated intracellular OPN on CSC progression, we performed RNAseq on CSCs from 5-months-old DEN-injected WT and *Opn*^{Hep} Tg mice. We identified that overexpression of *Opn* in CSCs activates multiple signaling pathways known to play a role in carcinogenesis (STAT3, HIPPO, PI3K/Akt and decreased p53 signaling). Unexpectedly, the effect of elevated OPN was very different in normal hepatocytes, with a decrease in mRNA signatures associated with carcinogenesis.

Last, we sacrificed DEN-injected mice from WT, *Opn*^{Hep} Tg, *Opn*$^{\Delta Hep}$ and, *Cd44*$^{-/-}$ and *Cd44*$^{-/-}$ *Opn*^{Hep} Tg mice at 12 months. *Opn*^{Hep} Tg showed increased number of tumors. *Opn*$^{\Delta Hep}$ mice also had increased tumor burden, as shown by increased number of tumors >3 mm and higher liver-to-b. wt. ratio, despite no significant difference in the total number of tumors. Ablation of *Cd44* was not protective and there was no difference in the number of tumors, tumors >3 mm and liver-to-b. wt. ratio) between WT and *Cd44*$^{-/-}$ as well as between *Opn*^{Hep} Tg and *Cd44*$^{-/-}$ *Opn*^{Hep} Tg mice **(Figure 5)**.

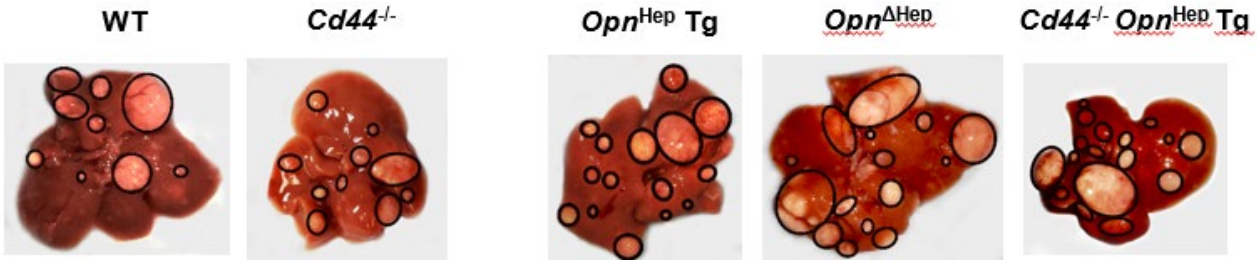

Figure 5. Gross appearance of livers from mice injected DEN and sacrificed 12 months later.

Conclusion: these results demonstrate that both ablation and overexpression of *Opn* in hepatocytes promote carcinogenesis. This is independent from CD44 and involves intracellular rather than secreted OPN. Ablation of *Opn* reduces the response to DNA damage and promotes the early arising of CSCs. This suggests a previously unsuspected protective role for OPN in hepatocytes at physiological levels. Unfortunately, the mechanisms involved in this process could not be unveiled in our study. Overexpression of *Opn* in hepatocytes does not affect the arising of CSCs but promotes their progression to cancer by activating multiple signaling pathways involved in carcinogenesis. We demonstrated that it does not involve OPN secretion and subsequent activation of a receptor.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Dr Desert worked as postdoctoral fellow on this project and did most of the experiments during the reported period. He worked closely with Dr Nieto that directed the project and was trained by Mr. Lantvit on how to handle mice and establish the HCC model. This project was for him a great experience that strongly enriched his skills in basic biology, biochemistry, animal models, data analysis and globally improved his knowledge in the field of HCC biology. He also presented his data at several conferences and seminars.

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Nothing to report

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Our results highlighted that intracellular hepatocyte derived OPN is a regulator of the early response to DNA damage, CSCs emergence and progression to cancer. We also demonstrated that OPN secretion and subsequent CD44 activation is not involved. We unveiled a previously unsuspected protective role for hepatocyte-derived OPN under physiological conditions. Even if the exact mechanisms could not be identified, this could lead to better understanding of the molecular mechanisms involved in liver carcinogenesis and potentially improve the therapies and/or the monitoring of HCC in the future.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to Report

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report

5. CHANGES/PROBLEMS: *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Many of the results from our different experiments were not aligned with our initial hypothesis (limited role of secreted OPN, p53 and CD44, protective role of OPN under physiological levels). This led us to use alternative approaches and hypotheses from what was initially planned (RNAseq, DNA methylation assay) in order to explore the potential mechanisms.
Key experiments involving CSCs isolation, culture and transplantation were also delayed due to difficulty to achieve reproducible results. This was solved over time.
Finally, the COVID19 pandemic also significantly delayed some of our experiments, making us to request a no-cost extension of the grant.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

The COVID-19 pandemic delayed some of the experiments and the breeding of mice.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Intracellular hepatocyte-derived osteopontin drives the onset of hepatocellular carcinoma. Romain Desert, Xiaodong Ge, Zhuolun Song, Hui Han, Daniel Lantvit, Wei Chen, Sukanta Das, Dipti Athavale, Ioana Abraham-Enachescu, Chuck Blajszczak, Yu Chen, Orlando Musso, Grace Guzman, Yujin Hoshida and Natalia Nieto. *Hepatology Communications*. 2021. Under review.

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Osteopontin Takes Center Stage in Chronic Liver Disease. Song Z, Chen W, Athavale D, Ge X, Desert R, Das S, Han H, Nieto N. *Hepatology*. 2021. Published.

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

- Hepatocyte-derived Osteopontin drives the development of hepatocellular carcinoma. Dr. Gary Kruh Cancer Research Symposium 2019. April 2019. UIC Student Center West. Chicago. Poster presentation. - Ablation of Osteopontin in hepatocytes drives the early onset of hepatocellular carcinoma due to DNA hypomethylation. The Liver Meeting 2020 - AASLD, online. Poster presentation. - Intracellular hepatocyte-derived osteopontin drives cell fate during hepatocarcinogenesis. Dr. Gary Kruh Cancer Research Symposium 2020. April 2019. UIC Student Center West. Chicago. Poster presentation.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

We have generated a new mouse model overexpressing *Opn* in hepatocytes in addition to global ablation of *Cd44* (*Cd44*$^{-/-}$ *Opn*^{Hep} Tg). In the future, this model could also be used to study the role of the OPN/CD44 axis in hepatocyte on any other liver related disease.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Name: *Romain Desert*
Project Role: *Postdoctoral fellow*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *36 months*
Contribution to Project: *Ms. Desert has injected and sacrificed the mice. He actively maintained and bred the mouse colonies. He analyzed the samples to address specific aim 1 and 2. He presented the data at conferences and seminars and contributed to writing and editing the manuscripts.*

Name: *Daniel Lantvit*
Project Role: *Technician*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *12.6 months*
Contribution to Project: *Ms. Lantvit assisted in maintaining and breeding the mouse colonies and provided technical assistance to sacrifice mice.*

Name: *Natalia Nieto*
Project Role: *Principal Investigator*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *5.4 months*
Contribution to Project: *D. Nieto directed the project, analyzed data and contributed to writing and editing the manuscript.*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to report

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ers.amedd.army.mil*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

9. APPENDICES:

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.